The function of potassium and sodium transporters in the maintenance of cellular ion homeostasis in rice (*Oryza sativa. L*) under hostile environmental conditions

By

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Declaration of Originality

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June 2017

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Dedication

To my parents:

Thank you for your love, precious support, and the sacrifices that you have made to give me my education. Please accept my deep everlasting gratitude and love.

To my brothers and sisters:

Thank you for your encouragement, invaluable advice, financial and technical support, and affection. To you I owe my deepest respect and esteem

To my wife Suha Alhomsi who has always supported me, emotionally and physically, during my studies, I cannot find the words to express the depth of my love and appreciation.

To my kids Rahaf and Adam:

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Abstract

The world population is growing alarmingly fast and imposes more pressure than ever towards increasing food production. However, this task is restrained by various abiotic stresses. One of these stresses is salinity. Salinity affects 230 million ha of irrigated land and results in over $27B in annual losses to agricultural sector. Salinity also poses a major threat to the food security, significantly reducing plant growth and yield. This reduction is especially pronounced in salt sensitive glycophytic crops such as rice. Rice is a staple food of more than half of the world population since as much as 20% of the world's dietary energy is supplied by it. Salt stress tolerance in plants is a multigenic and physiologically complex trait which is dependent upon a numerous cross-interacting mechanisms involving an orchestrated series of molecular, cellular, metabolic and physiological responses. In order to perceive, and respond to abiotic stress, plants have evolved a complex CBL-CIPK signaling network, which is Ca^{2+}-dependent and generate the secondary messengers, such as Ca^{2+} and reactive oxygen species (ROS). This signaling network initially alters protein activity within a cell, such as the activation of ion transporters and transcription factors. It is these interactions which then may up- or down-regulate the expression of responsive genes to induce adaptive responses. One such adaptive response is the plant's ability to retain or take up K^+, one of the most abundant and essential inorganic nutrients. Some members of the high affinity K^+ transporter (HAK) family are suggested to be involved in K^+ homeostasis under K^+ deficiency condition, and salt stress has also been found to alter their transcript levels. The Shaker-type AKT1 channel is another important component of the K^+ uptake system. It has been targeted by plant breeders in order to improve K^+ nutrition. Several studies have already reported that K^+ channels may mediate ion uptake of Na^+ and NH_4^+, particularly under a K^+-deficient conditions. Given that salinity stress results in acute K^+ deficiency, understanding of regulation of K^+ transporters under stress conditions may open prospects for genetic improvement of rice and minimising NaCl-induced yield losses.

Sodium exclusion from uptake and control over long-distance transport of Na^+ within plants are two components of a vital mechanism that plants have evolved to mitigate the adverse effects of salinity. The function of the high affinity potassium transporter HKT1;5 in the retrieval of Na^+ from shoots has already been identified in many species including rice.

The overall aim of this work was to elucidate the physiological role and functional expression of some of above ion transporters and signal transduction components (OsCIPK9, OsHAK1, OsHAK5, OsAKT1, and OsHKT1;5) in rice plants under stress conditions. This was achieved in a series of electrophysiological and whole-plant experiments conducted on the loss- or gain-of function rice mutants.

The first experimental chapter identified the critical regulatory role played by the calcineurin B-like protein-interacting protein kinase 9 (CIPK9) in rice plants, particularly under the K^+-deficient condition (e.g. mimicking the situation when K^+ availability is reduced in salinized soils). It was found that loss of function of *Oscipk9* increased plant sensitivity under the K^+-deficient condition. Moreover, under salt stress, the *Oscipk9* mutant plants had a lower K^+: Na^+ ratio than the wild type. In response to oxidative treatment (one of components of salt stress), the root of *Oscipk9* mutant plants grown under a low Ca^{2+} and K^+ condition experienced higher K^+ efflux compared to the wild type. This negative influence on the K^+ efflux was mitigated by an increase in the external Ca^{2+} concentration in the growth medium. However, when the rice plants were grown in a medium containing a high K^+ concentration, no significant difference in K^+ flux was observed between the lines. Therefore, these results suggest that OsCIPK9 plays a critical role in regulation of K^+ homeostasis under the K^+-deficient conditions.

The second experimental chapter investigated the role of HAK transporters in maintaining K^+ homeostasis during salt stress by studying the responses of rice (*Oryza sativa*) *Oshak1* and *Oshak5* mutants to salt and oxidative stresses. It was found that the loss of function of the *Oshak1* and *Oshak5* mutants caused (1) an increase in the amount of K^+ loss from

roots both under the K^+ deficiency (200 µM KCl) and saline (40 mM NaCl) condition, and (2) a decrease in the expression level of the respiratory burst oxidase homolog *OsRboH* genes in plants exposed to low K^+. This decrease attenuated stress-induced K^+ loss from *Oshak* mutants as compared with the WT. It is concluded that the loss of function of *Oshak1* and *Oshak5* increased the sensitivity of the mutant lines to salt stress, thereby revealing the crucial role of OsHAK1 and OsHAK5 in K^+ acquisition in response to hostile environmental conditions, such as low potassium and high soil salinity.

The third experimental chapter was focused used gain- and loss-of-function of *OsAKT1* gene to reveal the role of AKT1 channel on the ability of rice plants to uptake Na^+, NH_4^+ and K^+ ions. The results showed that in the presence of NH_4^+ the loss of function in *akt1* did not change the growth rate of the mutant plants. Furthermore, mutation in the *akt1* altered the Na^+ and K^+ content of the mutant plants, but had no significant effect on their growth. It was also found that AKT1 mediated Na^+ uptake, both under the K^+-deficient and low external NH_4^+ concentration condition. Additionally, the study also identified the role of the AKT1 channel in NH_4^+ uptake as it was found that increasing the external K^+ concentration until it was in the millimolar range stimulated stronger NH_4^+ influx in the elongation zone of the overexpressor compared to the wild type. Under K^+-deficient conditions, the presence of 2mM NH_4^+ inhibited AKT1 permeability to Na^+ ions in the overexpressing line. Overall, this study has proven that AKT1 plays an important role in ion homeostasis in response to hostile environmental conditions, particularly under K^+-deficiency.

The fourth experimental chapter has assessed the physiological role of OsHKT1;5 in the xylem Na^+ unloading in response to salt and drought stresses by comparing knocked-down *OsHKT1;5* (KD) line with its wild type. The phenotyping experiments showed that knocking-down of *OsHKT1;5* expression resulted in excessive level of Na^+ accumulated in the mutant plants KD under severe salt stress (80 mM NaCl), but much smaller amounts of K^+ accumulated in its

shoots. As a result, its growth was significantly impaired. However, the electrophysiological data showed that the KD plants had a greater capacity for exclusion of Na^+ from the elongation cells accompanied by a lower K^+ uptake. This difference has resulted from higher activity of the H^+-ATPase in the KD line. Such activation may also have enhanced the activity both of SOS1 (the Na^+/H^+ antiporter), and the HAKs K^+/H^+ symporter genes in the epidermal cells of the elongation zone of rice roots. Knocking-down of *OsHKT1;5* expression did not alter the growth of the mutant compared to the wild type during drought stress, suggesting OsHKT1;5 has no role to play during drought stress.

Overall, the finding of these studies has elucidated the crucial roles of the OsCIPK9 signaling network, the high-affinity K^+ transporters (HAKs), the AKT1 channels and the Na^+ transporter (HKT1;5) in ions homeostasis in rice plants in response to abiotic stresses. The information obtained from these studies could be used for further breeding program or to produce new lines in order to study the interactive effects of ion transporters in rice plants.

Abbreviation

ABA	Abscisic acid
AKT1	Arabidopsis potassium transporter 1
ATP	Adenosine triphosphate
B	Billion
BSM	Basal salt medium
CaMs	Calmodulins
CAT	Catalase
CAX1	H^+/Ca^{2+} antiporter
CBL-CIPK	Calcineruin-B-like protein-CBL-interacting protein kinase
CCC	Cation-Cl co-transporter
CDPKs	Ca^{2+}-dependent protein kinases
DAPCs	Depolarisation-activated potassium channels
DJ	Dongjin wild type
EC	Electrical conductivity
EDTA	Ethylene diamine tetra acetic acid
ENSO	El nino southern oscillation
ESP	Exchangeable sodium percentage
EZ	Elongation zone
G	Glycine
GI	Germination index
GORK	Guard cells outward rectifying potassium channels
Gs	Stomatal conductance
ha	Hectare
HAK	High-affinity potassium transporter
HATS	High-affinity potassium transport system
HKT	High-affinity potassium transporter
HY	Hawayoung wild type
KO	Knock-out
KOR	Potassium outward rectifying channel
LAI	Leaf area index
LATS	Low-affinity potassium transport system

LIX	Liquid ion exchanger
MIFE	Microelectrode ion flux estimation
MP	Membrane potential
MZ	Mature zone
NHX1	Na^+/H^+ exchanger
NO	Nitric oxide
NSCCs	Non-selective cation channels
OX	Overexpressor
PCD	Programmed cell death
PCR	Polymerase chain reaction
PD	Pore domain
PEG	Polyethylene glycol
PLP	pyridoxal 5-phosphate
POD	Peroxidase
Rboh	Respiratory burst oxidase homolog protein
RGR	Relative growth rate
ROS	Reactive oxygen species
S	Serine
SKOR	Stelar outward-rectifying potassium channel
SNF1	Sucrose non-fermenting-like 1
SOD	Superoxide dismutase
SOS1	Salt overly sensitive 1
TPK	Two pore potassium channel
WT	Wild type

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Chapter 1

General introduction

1.1 Salinity problem

Abiotic stresses, such as drought and salinity, result in significant losses in plant production, worldwide. Global warming and population growth all contribute, indirectly, to these stresses (Koyro et al., 2010). A number of climate models predict that global warming will increase the world's average temperature by 1.4 - 5.8°C during this century (Thornes, 2002). Worldwide, areas that experience low rainfall have more than doubled in size since the 1970s, with the largest increase occurring in the early 1980s, due to the effects of El Nino Southern Oscillation (ENSO), which caused reduced precipitation and increased surface warming (FAO, 2009). Another contributing factor has been the 2016 world population which is expected to have increased by more than 2.3 billion people, by 2050. Therefore, this trend means that global food production, especially cereal crops, will have to increase by 70% over current outputs to meet the demands of the expected increase in population numbers (FAO, 2009). However, food production is currently facing two major challenges: firstly, land is becoming scarcer, due to increased urbanisation, with a growth of 21% in urban areas predicted by 2050. Secondly, water supplies have fallen to an alarming level in a considerable number of countries (FAO, 2009). Thus, the world still has a long way to go to meet the food demands, not only of a significant proportion of the current population, but also of the increased population of the future. Thus, in order to meet both current and future global demands for food, increased plant productivity in arid and semi-arid lands by cultivating crops with high salt tolerance is essential. More than 6% of global land, or 831 million hectares, is salt-affected by either salinity or sodicity (Martinez-Beltran and Manzur, 2005). Australia, with an agricultural area of about $7.6x10^6$ km^2 is considered to be the most salt-affected of the continents with approximately one-third of the world's salt-affected soil, and approximately half

(over 60% of soils in agricultural areas) of the world's sodic-affected soil (Szabolcs, 1989). Annually, salinity issues cost the Australian economy more than $1.6 billion (Rengasamy, 2002).

1.2 Classification of salt-affected soil

The properties and attributes of salt-affected soil have been known for several decades (Fao, 1988; Nrcs, 2010; Richards, 1954; Soil Survey Division, 1993). According to Richards (1954) saline soil is characterised by a high level of sodium salts, electrical conductivity of saturated soil extract (ECs ≥4 dS m^{-1}, pH ≤ 8.2), an Exchangeable Sodium Percentage (ESP ≤ 15%) and typical soil structure. Sodic soils contain low soluble salts (ESP ≥ 15%) with a pH range of between 8.5 and 10, and poor soil structure (Richards, 1954), while saline-sodic soils are characterised by significant amounts of soluble salts (ECs ≥4 dSm^{-1}) and exchangeable sodium (ESP ≥ 15% and a pH ≤ 8.5).

In terms of its causes, salinity can be classified into primary (or natural) salinity and secondary (or human-induced) salinity. Primary salinity is caused by the geochemical weathering of rocks, sediments and soil, which produce dissolved mineral salts thataccumulate over a long period of time in the soil and groundwater, lakes, and marshlands (Gabrijel et al., 2011; Tanji, 2004; Williams, 1999). In arid and semi-arid areas where salinity is a considerable problem, these dissolved minerals salts are the result of a high evaporation to precipitation ratio (Tanji, 2004). In these areas, genetic improvement of plants is the only option for increased crop production (Shabala and Munns, 2012).

There are a number of other possible natural causes of salinity in soils: (1) seawater intrusion into coastal areas due to excessive groundwater pumping, (2) accumulation of dissolved minerals salts in the subsoil due to a rise in groundwater, resulting from evaporation (Gabrijel et al., 2011; Rengasamy, 2006; Shabbir and Khalilur, 2010; Williams, 1999).

Secondary salinity refers to human activity (anthropogenic factors), including ‘dryland agriculture’ (Halvorson and Richardson, 2011) or ‘hydrologic imbalance’ (Shabbir and Khalilur,

2010), which is the result either of the loss or removal of native vegetation in favour of crops and pastures that have different water-use requirements, or of overgrazed pasture lands. In this case, groundwater rises due to a high precipitation to evapotranspiration ratio. In this water, mineral salts dissolve and are carried to the root zone where they impair plant growth and yield (Shabbir and Khalilur, 2010). Another important factor concerning increasing salinity is the excessive use of brackish irrigation water over long periods of time, resulting in the groundwater rising into the rhizosphere, causing salinisation and water-logging (Tanji, 2004). In Australia, the amount of agricultural land affected by dryland salinity in 1996 was estimated at 25000 km^2 (Robertson, 1996) with more than half of that area being located in the south-west of Western Australia (Government of Western Australia, 2000). Furthermore, it is predicted that the size of this affected area could possibly increase to 170000 km^2, within 50 years (National Dryland Salinity Program, 1998).

1.3 Plant diversity in terms of salt tolerance

Plants vary significantly in their salt tolerance. Four categories of crops have been identified by Mass and Hoffman (1977) on the basis of their relative yield during long-term exposure to salt conditions: tolerant, moderately tolerant, moderately sensitive and sensitive. Salt sensitive herbaceous crops have been ranked differently in various reviews. However, Tanji and Kielen (2002) reported that, based on collected data of many researchers, barley, canola, cotton, oats and sugar beet are the most tolerant of the cereal crops; sorghum and wheat are moderately tolerant and chickpeas, corn and alfalfa are moderately sensitive, while rice is the most sensitive. The terms ‘halophyte’ and ‘glycophyte’ have been introduced by scientists to describe plants with the ability to grow in a wide range of salt concentrations. ‘ Halophyte’ refers to plants that are able to complete their life-cycle in soil containing at least 200 mM of NaCl (Flowers and Colmer, 2008). A wide range of salt tolerance has been identified among halophytic plants. For example, a relative growth rate reduction occurs at 300 mM NaCl in *Aster tripolium* at 375 mM in *Beta*

vulgaris ssp. *Maritime* at 500 mM, in *Spartina townsendii*, and at 750 mM in *Sesuvium portulacastrum* (Koyro et al., 2010).

There may be substantial physiological and genetic variations between the dicotyledonous and monocotyledonous species. While the former exhibits a significant relative growth rate, that of the latter is less dramatic in response to saline conditions (Shabala and Munns, 2012). Under NaCl conditions ranging from 200 to 360 mM, the relative growth rate for dicotyledonous halophytes was between 6 and 160 mg g^{-1} d^{-1}, while for monocotyledonous halophytes it was 6 and 26 mg g^{-1} d^{-1} (Debez et al., 2006; Flowers and Colmer, 2008). In contrast, glycophytic plants respond to relatively low salt concentrations by restricting salt translocation from root to shoot, and are, therefore, classified as salt excluders. Comparing morphological, physiological, and biochemical mechanisms between or within species is important for identifying and understanding the mechanisms that allow plants to adapt to water and salinity stress.

1.4 Major constraints imposed by salinity

1.4.1 Osmotic stress

Salt concentrations that have increased above the threshold level in the rhizosphere reduce the osmotic potential of the soil solution, thus, limiting the water uptake by plant roots, and so causing initial osmotic stress (Munns and Tester, 2008). This results in a non-functional turgor potential, a lower rate of leaf expansion and a slower development of photosynthetic leaves (Munns, 2011) due to partial or complete stomatal closure and a lower photosynthetic rate. The salt threshold varies according to the genotype, the variety and the growth stage of the plant; for example, in salt-sensitive species such as rice, the threshold is approximately 30 mM NaCl, while in salt tolerant species such as barley, it is 80 mM NaCl (Mass and Hoffman, 1977). However, a toxic concentration of Na^+ is not well defined. Osmotic stress can be partially managed through the process of osmotic adjustment and osmoprotection, whereby compatible solutes are accumulated in the cytosol and organelles of plants (Neumann, 2011). These osmolytes can be

essential elements, such as K^+, which are absorbed from the medium, or organic solutes, such as sugars, sugar alcohols, amino acids and amines (Peleg et al., 2011). The beneficial feature of these solutes is that plants can accumulate high levels of them without risking interference with their normal cellular metabolism (Bohnert and Jensen, 1996). In contrast, the synthesis of compatible solutes is a rather slow process with a time-scale measured in hours (Verbruggen et al., 1996) or even days (Kishor et al., 1995), and ATP is required for it to occur (Munns and Tester, 2008). For example, to maintain vacuolar osmolarity in a plant shoot, seven moles of ATP are required to accumulate one mole of NaCl or KCl, four moles for transfer from root to xylem, and three moles from xylem to shoot cell vacuoles (Raven, 1985). In contrast, the ATP requirements for synthesis and accumulation of organic solutes is significantly higher; for example, 71.4, and 81.5 moles of ATP are needed to accumulate one mole each of proline, and glycine betaine, respectively, in plant shoot cells with NH_4^+ as the N source (Raven, 1985). This process comes at the expense of plant growth (Munns and Gilliham, 2015) and this is more so in glycophytes than halophytes. Halophytes use inorganic salts as their main internal osmoticum, while glycophytes tend to exclude Na^+ and Cl^-, instead using absorption ions in the roots and stem as the main mechanism for preventing excessive accumulation of salt in photosynthetic tissues. Flowers and Colmer (2008) found that inorganic solutes of Na^+ and Cl^- constituted 67% of the total solute concentration in 32 species of chenopodiaceae (halophytes), whereas the percentage was only 32% in 17 species of poaceae; in contrast, sugar concentrations were 1%, in chenopodiaceae, and 19% in poaceae (glycophytes).

1.4.2 Ionic stress

The presence of high concentrations of Na^+ and Cl^- in the rhizosphere can restrict plant growth by causing nutrient imbalance and ion toxicity. Nutrient imbalance may result from the effects of salinity on nutrient availability, competitive uptake, transport or partitioning within the plant, or an increase in plant nutrient requirements due to the physiological inactivation of nutrients

(Grattan and Grieve, 1998). For example, excessive levels of Na^+ in the cytosol is toxic to a plant cell, as Na^+ competes with K^+ at the sites in transporters and enzymes that normally bind the essential cation K^+. Such competition is due to the physicochemical similarities between them (Maathuis and Amtmann, 1999). Ion toxicity is the result of ion accumulation exceeding the ability of vacuoles to compartmentalise the ions. In glycophytes, salts can accumulate rapidly in the cytoplasm, thereby inhibiting enzyme activity, or causing cell dehydration due to its accumulation in the cell walls (Koyro et al., 2010).

It has been reported that the ability of a plant to maintain a low cytosolic Na^+:K^+ ratio is a critical factor in determining its salt tolerance. Reducing the entry of salt to the roots and its transport into the plant, as well as reducing salt concentration in the cytoplasm by compartmentalising it in the cell vacuoles, are considered the main mechanisms for maintaining non-toxic levels of Na^+ and Cl^- ions in the cytoplasm (Koyro et al., 2010; Maathuis and Amtmann, 1999; Munns, 2011).

High Na^+ concentrations can be avoided by a mechanism called an excluder. (Munns, 2011) reported that most plants exclude 98% of the salt absorbed by the roots into the rhizosphere, thus reducing the ions that are translocated to the shoot to as little as 2%. However, since the exclusion of cytosolic Na^+ to the rhizosphere is a thermodynamic process, it requires energy (Shabala and Munns, 2012). There are several genes play a crucial rule in maintain low concentration of cytosolic Na^+. For example, The Na^+/H^+ antiporter, SOS1, which was identified as a Na^+ excluder from root cells to soil surface. While in parenchyma cells, SOS1 is responsible for Na^+ loading into the xylem sap (Hanin et al., 2016). Whereas, SOS1 is essential to exclude Na^+ out of cells, the NHXs are required to maintain low Na^+ in cytosol via acquired Na^+ within the vacuole (Deinlein et al., 2014). The other excluder genes are explained in the section 1.7.3.5

1.4.3 Oxidative stress

In plants, poor osmotic adjustment may lead to turgor loss, and stomatal closure that is quickly followed by reduced gas exchange and decreased photosynthesis. This, in turn, causes exposure of chloroplasts to excessive excitation energy, impairing the cellular electron transport system and resulting in increased production of reactive oxygen species (ROS) (Pallavi et al., 2012), such as superoxide anion ($O_2^{-\bullet}$), hydroxyl radical ($^{\bullet}OH$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$) (Miller et al., 2010; Pallavi et al., 2010). During this process, an imbalance of ROS and their antioxidant systems can occur, causing oxidative stress. ROS production can take place in different organelles, including the chloroplast, peroxisomes, mitochondria and the apoplast. Multiple roles have been identified for ROS: they play a role in the biosynthesis of lignin (Denness et al., 2011; Gill and Tuteja, 2010) and they act as signal transduction molecules for sensing the effects of adverse environmental conditions (Demidchik, 2010; Gill and Tuteja, 2010; Miller et al., 2010; Pallavi et al., 2010). For example, quick activation of Ca^{2+} permeable channels, by a process called K^+/electrolyte leakage, causes a rise of Ca^{2+} in the cytosol (Demidchik et al., 2007) and activation of K^+ efflux channels (Demidchik et al., 2003). In addition, excessive levels of ROS can cause oxidative damage to fundamental cellular components, including membranes, proteins and nucleic acids, and, eventually, can activate programmed cell death (PCD) (Pallavi et al., 2010). In order to mitigate this oxidative damage, excessive levels of ROS are removed during the detoxification process that is effectively achieved by two types of anti-oxidative system: a non-enzymatic system, such as ascorbate, phenolic compounds, glutathione, tocopherol, carotenoids, and proline, and an enzymatic system, such as superoxide dismutase (SOD), Catalase (CAT) and peroxidase (POD) (Demidchik, 2010; Gill and Tuteja, 2010; Pallavi et al., 2012; Pallavi et al., 2010). Change in the activity of antioxidant enzymes and the levels of some non-enzymatic antioxidants were assessed for their usefulness as markers of salt tolerance in different plant species (Pallavi and RamaShanker, 2010). When barley plants were exposed to H_2O_2, the salt–

tolerant varieties showed better oxidative stress tolerance due to a superior ability to acquire K^+. This suggests that, in barley, ROS-activated K^+-permeable channels are involved in salinity signalling and adaptation (Maksimovic et al., 2013). However, this study also reported that efficient salt and oxidative stress tolerance is not associated with higher enzymatic antioxidant activity (Maksimovic et al., 2013). Later, Adem et al. (2014) observed that the highest K^+ retention was found in the salt tolerant cultivar Numare, followed by Golden Promise, while the salt sensitive Naso Nijo had the highest K^+ efflux, suggesting that the resistance of barley's tissue to oxidative stress is critical in salt tolerance mechanism. In Arabidopsis, when the roots were incubated in exogenous compatible solutes, such a glycine, proline or mannitol, for fifty minutes prior to the exposure to oxidative stress, $OH^{\bullet}$-induced K^+ efflux was significantly reduced, with the large reduction recorded in those pre-incubated in glycine betaine (Cuin and Shabala, 2007). This study suggested that compatible solutes play a regulatory role, such as K^+ channel blocking in the case of glycine betaine, as well as ROS scavenging, thereby mitigating the adverse effect of oxidative stress (Cuin and Shabala, 2007). Similarly, the exogenous application of glycine betaine significantly ameliorated the response of quinoa (*Chenopodium quinoa*) to the oxidative stress, by minimising the negative impact of oxidative (UV-B) stress on PSII in a dose dependent manner, as well as its role in osmotic adjustment (Shabala et al., 2012). In barley and wheat, organic osmolytes also played an important role both in leaf osmotic adjustment and in the activity of PSII in leaves exposed to oxidative (UB-V) stress thus suggesting that oxidative stress tolerance is strongly related to salinity-induced accumulation of organic osmolytes in the leaves of barley and wheat plants (Puniran-Hartley et al., 2014).

A plant's ability to maintain a high antioxidant capacity in order to scavenge for the toxic reactive oxygen species (ROS), has been linked to increased salt tolerance in rice. (Fadzilla et al., 1997; Lin and Kao, 2000; Nounjan et al., 2012; Turan and Tripathy, 2013; Vaidyanathan et al., 2003). A study was carried out on two cultivars of *indica* rice (*Oryza sativia L.*) seedlings, the salt

sensitive cultivar Malviya-36, and the salt tolerant CSR-27 under conditions of 7, and 14 dS m^{-1} of NaCl, respectively The results showed that salt tolerance in indica rice seedlings is associated both with higher levels of the antioxidants ascorbate and glutathione, and higher activity levels of the anti-oxidative enzymes SOD, CAT, GPX, APX, and GR. This suggests that both antioxidants and anti-oxidative enzymes can be used as determinants of salt tolerance in Indica rice seedlings (Mishra et al., 2013). In another study, salt sensitive (MI-48, IR-28) cultivars and salt tolerant (CRS-1, Pokkali) cultivars of rice were used to assess salinity-induced oxidative stress and their anti-oxidative defense system. By increasing the salinity level, the production of the superoxide radical ($O_2^{-\bullet}$) and hydrogen peroxide (H_2O_2) increased significantly in the salt sensitive cultivars, and non-significantly in the salt tolerant cultivars. On the other hand, the activity of the superoxide dismutase (SOD) and also that of glutathione reductase (GR) declined in the leaves of the salt-sensitive cultivars, but increased in those of the salt-tolerant cultivars. Moreover, basal levels of catalase (CAT) and ascorbate peroxidase (APX) were much higher in the salt-tolerant cultivars. Salinity increased the concentration of CAT and peroxidase (POX) in all cultivars, but the increase was greater in the salt-sensitive cultivars than in their salt-tolerant counterparts. The ratio of glutathione-reduced (GSH) to glutathione-oxidised (GSSG) was also enhanced in the salt-tolerant cultivars under saline conditions. These results suggest that an increase in the activity of enzymes, such as SOD, CAT and GR, as well as a higher basal level of APX, accompanied by a high GSH/GSSG ratio, may enhance the performance of salt-tolerant cultivars compared to salt sensitive ones, under salt conditions (Chawla et al., 2013). Significant differences were found between the physiological and anti-oxidative responses of two varieties of rice (Pokkali and Nona Bokra) in response to 200 mM concentration of sodium chloride for 24, 48, and 72 hours. Results showed that Nona Bokra was a salt tolerant cultivar due to its lower Na^+ accumulation, minimal loss of K^+, higher chlorophyll and proline content, higher phenolics, lower lipid peroxidation, moderate free radical accumulation, and high GPX activity, compared to the salt-sensitive Pokkali

cultivar (Ghosh et al., 2011). In conclusion, it would appear that the maintenance of an efficient antioxidative system is vital to salt tolerance in rice (Moradi and Ismail, 2007). The effect of oxidative stress is summarised in (Fig. 1.1).

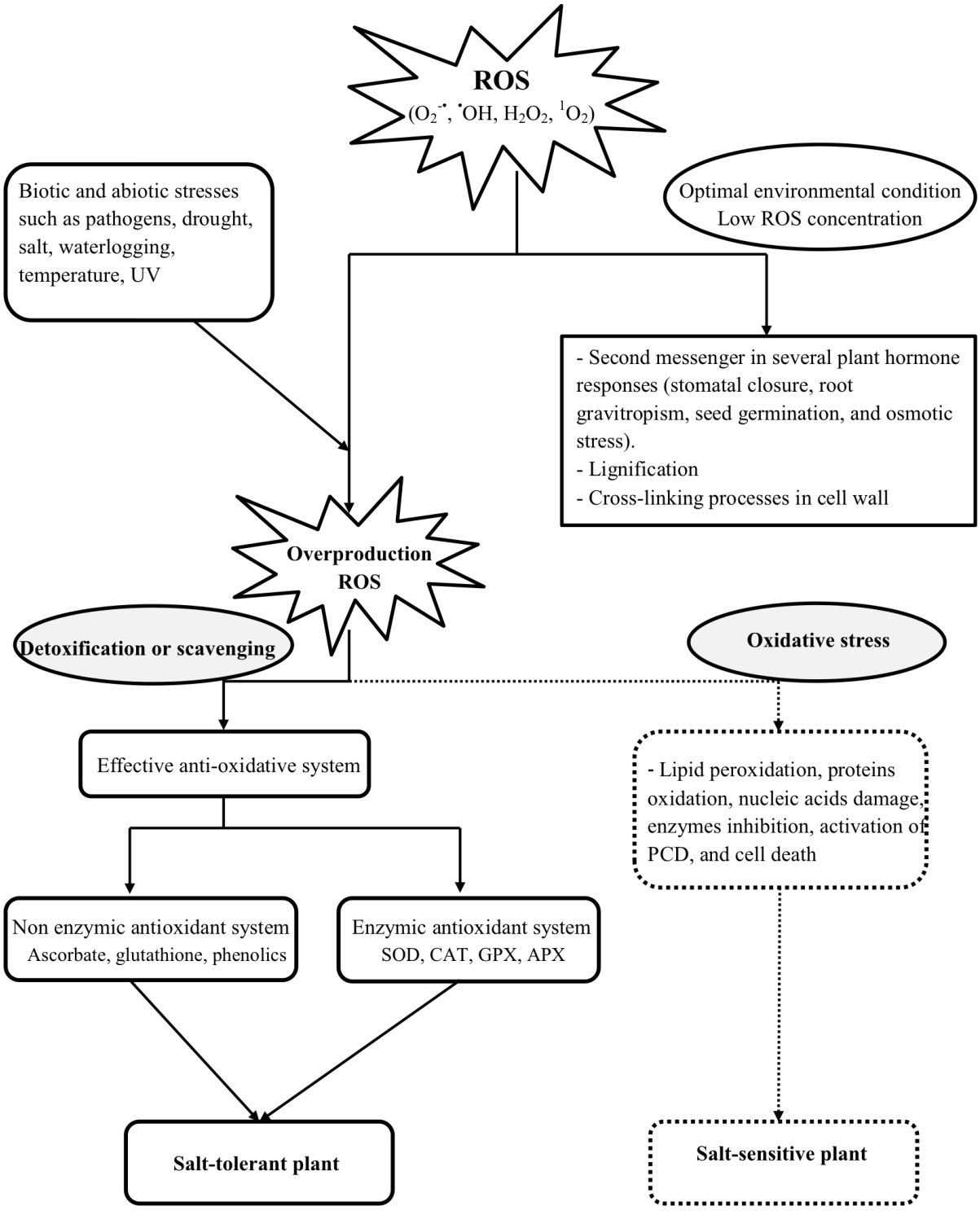

Figure 1. 1 Oxidative stress pathway, and plant responses (Demidchik, 2010; Gill and Tuteja, 2010; Miller et al., 2010; Pallavi et al., 2012)

1.5 Classification of the *Oryza* species

Rice is one of the most important staple foods being a major source of nutrition for approximately two-thirds of the world's population. Taxonomically, in genome AA, the genus *oryza,* belonging to the family poaceae, contains 22 species, of which 20 are wild species and two, *O. sativa* and *O. glaberrima,* are cultivated (Vaughan, 1994). *O. sativa,* the dominant rice species is grown in most countries of the world. Garris et al. (2005) detected five different sub-populations in *O. sativa L*: two in *indica* (*Indica* and *aus*) and three in *japonica* (*temperate japonica*, *tropical japonica*, and *aromatic*). *Indica* and *tropical japonica* cultivars are grown in most of Southeast Asia, whereas the *temperate japonica* cultivar is grown mainly in Japan, Korea, and Northern China (Doi et al., 2008).

Oryza sativa L. is generally considered to be a salt sensitive species, although natural variations in salt tolerance have been detected among its varieties (De Leon et al., 2015; Kanawapee et al., 2012; Sakina et al., 2016). Genotype variation was studied by Kanawapee et al. (2012) in 106 genotypes of rice (*Oryza sativa* L.) grown in 6 dS m^{-1} of NaCl for three days, and in 12 dS m^{-1} for seven days. Injury symptoms in the stressed seedlings were positively correlated both with the Na^+:K^+ ratio and the proline content, and negatively correlated with survival percentages and chlorophyll concentration. Based on the data collected, the genotypes were classified into five groups: 13 genotypes were considered tolerant, 13 were moderately tolerant, 33 were moderately sensitive, 28 were sensitive, and 7, highly sensitive.

1.6 Morphological, physiological and biochemical effects of salinity components on rice plants, during their different growth stages

1.6.1 Effect of salinity on growth stages of rice plants

Salt tolerance varies as a function of the growth stages of a plant (Lunin et al., 1963). Most research shows that while the majority of annual crops are salt tolerant at germination, they are salt sensitive at emergence and during their early vegetative growth (Läuchli and Grattan, 2011; Läuchli and Grattan, 2007). For example, rice is relatively salt-tolerant at germination (Heenan et al., 1988; Khan et al., 1997), but sensitive to salinity both during emergence and the early seedling growth stage. It then becomes tolerant, once more, during the tillering stage (Lutts et al., 1995), and again, during the reproductive growth stage (Khatun and Flowers, 1995; Zeng et al., 2001). However, no correlation between sensitivity at germination and other growth stages was found (Alam et al., 2004). A period of salt stress may affect the growth and yield of rice. A significant reduction was recorded in a number of filled spikelets, 1000-grain weight, and grain yield, when salinity was applied during the reproductive rather than the vegetative growth stage (Castillo et al., 2007). In a study carried out on two cultivars of rice, three levels of NaCl (1.8, 3.2 and 4.6 dS m^{-1}) were applied to each of five different growth stages, for 20 days: the seedling stage, 1-leaf, 3-leaf, panicle initiation (PI), and booting. The results indicated that, during the 20 day period between the 3-leaf and PI stages, the plant displayed a sensitivity to salinity, in terms of lower seedling yield (Zeng et al., 2001). This finding suggests that irrigating the plants after the booting stage with water containing a low to moderate concentration of NaCl has a significant effect on seed yield, while using poor water during the critical stages of 3-leaf and PI may cause a significant yield reduction. Thus, identification of a particular plant's salt sensitivity at different growth stages may improve saline water management (Zeng et al., 2001).

1.6.2 Germination

The process of germination is affected by the nature and duration of salinity. The process of germination was categorized by (Abdul et al., 2010) into: (1) imbibition of water, (2) active metabolism, (3) emergence and elongation of embryonic tissues, and (4) establishment of seedlings. They found that all four stages were affected by salinity. seed germination was reduced because salinity induces osmotic and ionic stresses (Wahid et al., 1998), which may cause a reduction in hydrolytic enzyme activity. This in turn breaks down the complex reserves of the seed into simple molecules that are needed for metabolic activities, such as cell division and elongation. As previously mentioned, it has been established that rice is least salt sensitive, during its germination stage. The effects of salinity on plant growth stages, in five varieties of rice, (Heenan et al., 1988) showed that salinity up to a maximum of 16.3 dS m^{-1}, had no significant effect on seed germination. However, germination was severely inhibited, in all cultivars, after 28 days of exposure to 22 dS m^{-1} of salinity. Another study, conducted on nine cultivars of rice, showed that they differed in their response to salinity (Khan et al., 1997). The germination index (GI) decreased as the salinity concentration increased. Under conditions of up to 150 mM NaCl, all cultivars had a more than 80% germination success rate, while, at 200 mM NaCl, germination was reduced by 50% in the majority of the cultivars. Furthermore, salinity delayed the emergence of seedlings (Khan et al., 1997). A recent study was carried out on 102 cultivars of rice, to evaluate their salt tolerance both during the germination and seedling stages. Under a 15 dS m^{-1} salt condition, seed germination ranged between 73% and 89%, while, at 20 dS m^{-1}, it was reduced significantly, to less than 75%, in all cultivars (Islam and Karim, 2010).

1.6.3 Vegetative and reproductive growth

Meristematic cell division and elongation are processes that are vital to plant growth. Excessive levels of salinity in the root zone causes a reduction both in cell and whole-plant growth (Neumann, 2011). According to Mass and Hoffman (1977) 3 dS m^{-1} is the threshold of salinity

that can be tolerated by rice plants before their growth and yield is affected. Indeed, the effect of salinity on the growth and yield of rice has been the subject of a number of intensive studies by numerous authors (Clermont-Dauphin et al., 2010; Garcia et al., 1995; Heenan et al., 1988; Lee et al., 2003; Yeo et al., 1990; Zeng and Shannon, 2000). For example, ten rice cultivars were evaluated for their tolerance to three different concentrations of salinity (4.5, 8.5, and 12.5 dS m^{-1}), and then compared to ones under a control condition (Alam et al., 2004). Plant growth was reduced at 4.5 and 8.5 dS m^{-1}, and then completely inhibited at 12.5 dS m^{-1}. Salt injury symptoms were distinctly visible on older leaves whose upper portions rolled up and withered away. Also, the emerging leaf blades were tightly rolled up, their tips severely withered and necrotic. However, the younger leaves remained succulent, and appeared to be an even darker green than the control. The treated plants displayed symptoms of nitrogen deficiency: tiller production was restricted by 20% at 4.5 dS m^{-1}, and by 70% at 12 dS m^{-1}, after which the plants gradually died (Alam et al., 2004). Plant height, leaf area, and both root and shoot fresh weight decreased as the salt concentration increased (Alam et al., 2004). However, a variety of responses was detected among the different cultivars (Alam et al., 2004; Cha-um et al., 2009). In salt-stressed seedlings grown under osmotic stresses, the degradation both of total chlorophyll and total carotenoids was positively correlated with the photosynthetic rate, and impaired plant growth (Cha-um et al., 2009).

The effect of salinity, on 80 cultivars of rice, was studied by Ali et al. (2004) using a mixture of four commercial salts (Na_2SO_4, NaCl, $MgCl_2$ and $CaCl_2$) in the ratio of 10:4:1:5. The results showed that, in all genotypes, plant yield, chlorophyll concentration, fertility percentage, and number of productive tillers, as well as the panicle length and number of primary branches per panicle, were reduced. Zeng et al. (2003) found a significant correlation between the leaf area index (LAI) and yield components of salt sensitive and salt tolerant cultivars. These results suggest that yield losses under salinity conditions could be the result of a reduction in leaf area index. A strong relationship between the physiological parameters, Na^+, K^+, Ca^{2+}, K^+-Na^+ selectivity ($S_{K,Na}$)

and Na^+-Ca^{2+} selectivity ($S_{Na,Ca}$) and growth performance as measured by the number of tillers, leaf area, plant height and shoot dry weight, was detected in 31 genotypes of rice grown in 0.9 and 6.4 dS m^{-1} of salinity consisting of NaCl and $CaCl_2$ in a 6:1 ratio. These genotypes were then classified into three groups, based on sodium content and K^+-Na^+ selectivity: group one, with high $S_{K,Na}$ and low shoot Na^+ content; group two, with intermediate $S_{K,Na}$ and shoot Na^+ content; and group three, with low $S_{K,Na}$ and high shoot Na^+ content (Zeng, 2005).

1.6.4 Photosynthesis

Osmotic stress induces stomatal closure followed by reduced gas exchanges and decreased photosynthesis (Shannon, 1997). In addition, the accumulation of toxic levels of Na^+ and Cl^- in the photosynthetic organs severely inhibits the activity of enzymes, including those involved in photosynthesis (Munns and James, 2006). The inhibition of gas exchange is mediated by shoot- and root-generated hormones (Chaves et al., 2009). However, it is a plant's ability to recover photosynthesis after salinity stress that determines its salt tolerance. The speed and extent of this recovery is dependent on the intensity and duration of the stress (Chaves et al., 2009). Stomatal conductance is essential both for the acquisition of CO_2 and the prevention of dehydration (Medici et al., 2007). Furthermore, it is known that exposure either to prolonged water stress or salinity, especially during plant development, causes profound modifications in leaf anatomy, such as thickened cell walls, and smaller and more densely-packed leaf cells (Longstreth and Nobel, 1979). This finding may explain the long-term reduction of mesophyll conductance (g_m) in salt stressed plants, and consequently, the inhibition of photosynthesis (Niinemets et al., 2009). However, factors such as the severity of stress exposure, as well as its duration and type, determine the relative contributions made by stomatal and non-stomatal components to limiting photosynthesis (Chaves et al., 2009). The function of photosynthesis and chlorophyll fluorescence was studied by Moradi and Ismail (2007) in salt tolerant rice cultivars (IR651 and IR632) and one salt sensitive cultivar (IR29), under 6 and 12 dS m^{-1} of salinity, both during the seedling and

reproductive stages. Photosynthetic CO_2 fixation, stomatal conductance (g_s) and transpiration, all decreased significantly as the salt concentration was increased, with the greater reduction occurring in the salt sensitive cultivar IR29. Here, stomatal conductance continued to decrease, with no subsequent recovery, while the salt tolerant cultivars IR651 and IR632 experienced rapid stomatal closure within the first four hours of salinisation, followed by a partial recovery after 72 hours. Although chlorophyll fluorescence measurements revealed no significant differences in the actual quantum yield of photosynthesis, a decrease in the electron transport rate was detected under salt stress.

1.7 Plant potassium channels

Different types of K^+ channels have been identified as being involved in K^+ transport in plants. These are Shaker-type, and Tandem-pore K^+-channels (TPK), and all types have orthologous in animals (Véry and Sentenac, 2003). The first plant K^+ channels identified through functional complementation of yeast mutant strains defective for K^+ uptake, were members of the Shaker family (Kv-like) (Schachtman et al., 1992; Sentenac et al., 1992). In the Arabidopsis genome, nine Kv-like Shaker proteins have been identified, seven of which were found to be highly selective K^+ channels (Very and Sentenac, 2002). Based on their rectification properties, these have been grouped into three known sub-families: four are inward-rectifying (KAT1, KAT2, AKT1, SPIK), two are outward-rectifying (GORK, SKOR), one a silent K^+ channel (KC1), and weak or unknown rectifying channels (AKT2/3, AKT5) (Pilot et al., 2003). The Shaker channels are voltage-gated; thus, changes in membrane polarisation, in response to the presence of K^+ in milimolar concentrations, regulate the functions of these channels (Lebaudy et al., 2007). The inward-rectifying channels are activated by membrane hyperpolarisation (negative E_k that is about -80 and -100 mV), so their gating is almost independent of the predominantly potassium concentration on both sides of the plasma membrane (Hedrich et al., 2011). However, since the outward-rectifying channels are activated by membrane depolarisation, their gating is, therefore,

strongly dependent upon the extracellular K^+ concentration (Hedrich et al., 2011; Lebaudy et al., 2007).

1.7.1 Expression pattern of Arabidopsis K^+ channel 1 (AKT1)

In Arabidopsis, AKT1 (locus ID At2g26650) is localised in the plasma membrane of the root, and through the use of gene promoter analysis, it was found that *AKT1* gene promoter activity was strong along the mature root, relatively weak at the root tip, and restricted both in the cap and epidermis (Lagarde et al., 1996). In rice plants, transcriptional analysis showed the presence of *OsAKT1* transcripts in the roots for K^+ uptake (Golldack et al., 2003). The highest of such transcripts was found in the epidermis, followed by exodermis, endodermis and the pericycle (Golldack et al., 2003). In leaves, the *OsAKT1* transcript was found in the mesophyll cells and cells neighboring the metaxylem vessels, suggesting that OsAKT1 plays a role in K^+ transport and distribution. Moreover, *OsAKT1* also expressed in phloem cells, suggesting its possible role in phloem loading in rice plants (Golldack et al., 2003).

1.7.1.1 Regulation of AKT1 channels

Responses to environmental stimuli, such as light, ABA, and salt stress, regulate the Shaker K^+ channels' activity at transcriptional, and post-translational levels, respectively (Chérel, 2004). For example, a study of the signaling pathway of nitric oxide (NO) in the *Xenopus* oocyte system and in the root protoplasts of Arabidopsis, showed that NO negatively regulated the activity of AKT1 *via* up-regulation of the activity of the vitamin B6 salvage pathway gene, SNO1. This caused an accumulation of B6 and pyridoxal-5′-phosphate (PLP), which in turn suppressed the activity of AKT1. Such suppression reduced the K^+ uptake by the root (Xia et al., 2014). Several studies showed that the activity of these channels is regulated by protein-protein interactions (Chérel, 2004), which can be grouped into two types: Firstly, those that form heteromeric channels. For example, a heteromerisation study showed that *AtKC1* modulates the activity of the root K^+

inward-rectifier, AKT1, under K^+-deficient conditions, by a combination of two effects. The first one is the integration of *AtKC1* into the root inward K^+ rectifier, which reduces K^+ efflux and results in membrane hyperpolarization by shifting the voltage-dependent gating of AKT1 to a more negative membrane potential. The second one involves reduction of the relative cord conductance by *AtKC1*. These two factors inhibit K^+ efflux while allowing K^+ uptake at negative membrane potential from reversal potential for K^+ (Geiger et al., 2009). Secondly, those that are interactions involving genuine regulatory proteins (Chérel, 2004). Several studies showed that *AKT1* is up-regulated by CBLs/CIPKs protein complexes in Arabidopsis (Xu et al., 2006), and in rice plants, where the calcineurin B-like protein OsCBL1 interacts with the protein kinase, OsCIPK23, in the plasma membrane, phosphorylating AKT1-mediated K^+ uptake by the root of the plants (Li et al., 2014). A recent study showed that both AtKC1 and CIPK23 synergistically modulate AKT1 activity under a low-potassium condition (Wang et al., 2016). This kind of interaction between AKT1 and the CBLs/CIPKs complex has already been discussed above, in Section 1.6.4.3.

1.7.1.2 The role of AKT1 in plant growth under various K^+ and NH_4^+ conditions

NH_4^+ and K^+ are both monovalent cations that influence each other both positively and negatively within the low to high-affinity K^+ transport range (Szczerba et al., 2008a). In rice, the growth of rice seedlings, and the accumulation of K^+ in plant tissue under the high affinity K^+ transport condition (0.02 mM K^+), were both significantly reduced in the presence of NH_4^+. However, when the rice plants were grown in NH_4^+, an increase in the external K^+ concentration caused them to show greater influx, translocation and tissue accumulation of K^+, accompanied by a reduction in NH_4^+ influx, resulting in a significant improvement in their growth (Szczerba et al., 2008a). One of the possible explanations for this is that, under a low-affinity K^+ condition NH_4^+ competes with K^+ for transport via the non-AKT1 component of the K^+ transport system (Spalding et al., 1999). It was demonstrated that the AKT1 component of the K^+ permeability was between 55 to 63% in the wild type under a low K^+ concentration of between 10 to 100 μM, and in the

absence of NH_4^+. However, in the presence of NH_4^+ seed germination, seedling growth and K^+ uptake were all inhibited in the *akt1* mutant plants (Spalding et al., 1999). Moreover, membrane depolarisation was also observed when plants were grown in the presence of NH_4^+ (Spalding et al., 1999). Such depolarisation is known to activate outward-rectifying channels, such as GORK, resulting in more K^+ efflux. Under 1.5 mM of external K^+ condition, K^+ influx was not affected by the presence of NH_4^+, while K^+ influx was stimulated by the addition of NH_4^+ in the low affinity K^+ range (40 mM K^+). However, under the high-nutrient supply, plant growth declined due to the energy consumption required to remove NH_4^+ and K^+ (Britto and Kronzucker, 2002; Szczerba et al., 2008a). An electrophysiological study concluded that AKT1 encodes the inward-rectifying channel responsible for K^+ uptake from root cells (Hirsch et al., 1998). The role of AKT1 in plant nutrition, studied by using reverse genetic screening, showed that, in a medium containing ≤ 100 µM K^+ concentration and in the presence of NH_4^+, *akt1* function in Arabidopsis plants was disrupted, causing significant growth inhibition, compared with wild type. This suggested that the NH_4^+ inhibits non-AKT1 K^+-uptake pathways, thereby making growth dependent on AKT1 (Hirsch et al., 1998). In contrast, the growth of the *akt1* mutant plants was slightly reduced at 1 mM K^+, compared to the wild type. Moreover, $^{86}Rb^+$ tracer flux analysis revealed that loss of function of *akt1* reduced the plants' ability to uptake K^+ from the medium (Hirsch et al., 1998). These results indicate that the AKT1 channel mediates potassium uptake in a growth medium containing a concentration of 10 µM K^+ (Hirsch et al., 1998).

Other studies, on *Brassica napus* and other species, showed that when the plants were grown in 0.5 mM and 5 µM of K^+, the K^+ content of the shoots and roots was significantly reduced, but without significant change in the plant biomass. However, this K^+-deficiency did not change the expression of the *AKT1* gene. This is consistent with the theory that low-affinity transporters mediate K^+ uptake in the millimolar range (Lagarde et al., 1996). A combination study of the function of AKT1 and AKT2 in roots Arabidopsis plants using knockout mutation and

electrophysiological assays showed that reduction in the K^+ permeability of the plasma membrane of root cells resulted in growth impairment in *akt1* seedlings. Moreover, in cotyledons, AKT1 was the major contributor to K^+ uptake, compared with AKT2 (Dennison et al., 2001). Additionally, under a low K^+ condition (10 to 100 µM range) and in the absence of NH_4^+, the growth of the wild type plants increased as the external K^+ concentration increased, indicating that, in the absence of NH_4^+, their growth rate was restricted by K^+ deficiency. Mutations in *akt1* and *akt2* non-significantly affected the growth rate of the plants under both of the above conditions. However, when NH_4^+ was added to the growth medium, the growth rate of the *akt1* mutant plants was inhibited, but not that of the *akt2* mutant plants., This negative effect of NH_4^+ on plant growth rate was mitigated by the addition of more K^+ to the growth medium (Dennison et al., 2001). The need for plasma membrane hyperpolarisation between -180 and -240 mV increased, under a low-potassium condition (10-100 µM) to allow the inward rectifying channels to mediate K^+ uptake (Geiger et al., 2009). Under a low-potassium condition, the loss of function of one of the essential root K^+ channels, such as AKT1, resulted in growth impairment (Geiger et al., 2009). A study involving a single *athak5* mutation and an *athak5akt1* double mutation in Arabidopsis plants showed that the growth of the *athak5* mutant plants was inhibited at 10 µM, but not at 20 µM K^+, while both the *athak5* and *akt1* double mutant plants failed to grow at 100 µM K^+, and experienced growth inhibition at 450 µM K^+. These results suggested that AtHAK5 and AKT1 are the major transporters in the high-affinity K^+ transport system, and that the *AtHAK5* gene is the most important component of the non-AKT1 pathway (Pyo et al., 2010).

Desbrosses et al. (2003) found that the root-hairs of the *akt1-1* mutant did not elongate when the plants were grown ≥ 10 mM. Thus, they concluded that AKT1 is required for root-hair elongation, in the 50-100 mM range of K^+ concentration. However, under zero-K^+ concentration the *akt1* mutant plants developed longer root hairs. Both of these results led them to the conclusion that AKT1 is a component of the low-affinity potassium uptake system in plants.

1.7.1.3 The role of AKT1 in Na^+ uptake under a low-potassium condition

It has been suggested that the AKT1 channel is involved in Na^+ uptake under a low-potassium condition (Golldack et al., 2003; Spalding et al., 1999). This suggestion arose from the finding that, under a low (10 µM) potassium condition, the growth rate of *akt1* mutant plants increased by 119% when the external Na^+ concentration was increased to 1000 µM Na^+, while this same growth rate was not stimulated by Na^+ at 100 µM K^+ (Spalding et al., 1999). A transcriptional study of the *AKT1* gene in salt sensitive rice (IR29), and also in salt tolerant lines, (BK, Pokkali), under a 150 mM NaCl condition, showed that while *OsAKT1* transcripts disappeared from the exodermis of the salt tolerant lines, their levels remained unchanged in the endodermis, and increased in the exodermis in the salt sensitive genotype. These findings point to *OsAKT1*'s being regulated differently in the various salt-dependent rice genotypes (Golldack et al., 2003). Moreover, this regulation of *AKT1* does not significantly affect potassium homeostasis under salt stress. Under 100 µM K^+ to low-mM K^+, the salt tolerant lines selectively excluded Na^+, while maintaining the cytosolic K^+. However, at low-µM K^+, these salt tolerant lines accumulated similar amounts of Na^+ as the salt sensitive one, suggesting a clear correlation between *OsAKT* expression and whole plant Na^+ selectivity (Golldack et al., 2003).

1.7.1.4 The role of AKT1 in adaptation to osmotic stress and stomatal movement

Potassium is considered to be a major inorganic osmoticum in plants, playing an important role in osmotic adjustment under drought conditions (Wang et al., 2013). Potassium uptake is partially mediated by voltage-gated K^+ channels located in the cellular plasma membrane (Maathuis et al., 1997). Under water stress conditions, and with a 1.4 mM K^+ concentration in a hydroponic system, shoot and root K^+ content was lower in *akt1* mutant plants than in the wild type, while at 10 mM K^+, the *akt1* plants did not show any K^+-dependent phenotypes. This result suggested that under 1.4 mM external K^+, the non-AKT1 components were not able to compensate for the loss of function of *akt1*, resulting in a lower K^+ content (Nieves-Cordones et al., 2012).

One of the essential adaptations that plants need to develop in order to survive drought stress, is a rapid stomatal closure to prevent dehydration (Wang et al., 2013). K^+ inward/ outward flux from guard cells via voltage-gated channels plays a crucial role in stomatal movement. Four inward-rectifiers, KAT1, AKT1, AtKC1 and AKT2/3, and one outward-rectifier, GORK, have been identified in the protoplasts of guard cells in the wild type of Arabidopsis (Szyroki et al., 2001). Moreover, patch clamp analysis on enzymatically-isolated guard cell protoplasts showed that KAT1 is the dominant inward K^+ channel, being responsible for 79% of K^+ conductance, while the other inward-rectifiers, collectively, deal with the remaining 21%. This leads to the conclusion that inward-rectifying K^+ currents in guard cells are mediated not only by KAT1 homomers, but also by the AKT1, AKT2, and AKT2/3 K^+ channels (Szyroki et al., 2001).

Nieves-Cordones et al. (2012) reported that, both in hydroponic and soil systems, *akt1* mutant plants sustained a significantly lower percentage of water loss, and experienced a lower transpiration rate, at 10 mM K^+ and under water-stress conditions,. Additionally, the efficiency of stomatal closure in the *akt1* mutant plants was higher in response to ABA, suggesting that disruption of *akt1* enhanced the plants' response to water stress.

1.7.2 The KT/HAK/KUP potassium transporter family

The *KT/HAK/KUP* family has been identified, firstly, as a K^+ uptake permeases (KUP) in bacteria K^+ (Schleyer and Bakker, 1993), and as a high-affinity potassium transporter (HAK) in fungi (Bañuelos et al., 1995). Several homologues have also been identified in plants, such as Arabidopsis (Maser et al., 2001), rice (Bañuelos et al., 2002; Gupta et al., 2008; Yang et al., 2009), maize (Zhang et al., 2012), and poplar (He et al., 2012). The ubiquitous presence of KT/HAK/KUP transporter genes in ancestral plant genomes infers their important roles in K^+ acquisition during plant growth, development, and adaptation to stress (Grabov, 2007). A considerable number of studies showed that KT/HAK/KUP transporter genes are involved both in low- and high-affinity K^+ transport, which overlap with the activity of K^+ channels (Bañuelos et al., 2002). The existence

of different genes with similar K^+ affinity can be explained by the fact that KT/HAK/KUP transporter genes mediate active K^+-H^+ transport (Rodriguez-Navarro, 2000), while the other transporters mediate uniport transport to maintain electrochemical equilibrium (Bañuelos et al., 2002). A study of the phylogenetic tree of Arabidopsis identified 13 genes of the *KT/HAK/KUP* family that exhibit strong similarities (Maser et al., 2001). One of these genes, *AtHAK5,* has been identified as a high-affinity K^+ transporter in Arabidopsis (Rodríguez-Navarro and Rubio, 2006). In the rice genome, 27 genes have been identified, distributed among eight rice chromosomes. Twenty of these, including *OsHAK2, OsHAK3*, *OsHAK7* and *OsHAK9,* have been shown to be localised in the plasma membrane, while the rest are located in four different organelles: *OsHAK10* in the tonoplant, *OsHAK14* in the mitochondrial inner membrane, *OsHAK15* in the chloroplast thylakoid membrane and AtKUP/HAK/K12 in the chloroplast membrane (Gupta et al., 2008). However, based on aphylogenetic analysis of 13 Arabidopsis and 27 rice genes, the *KT/HAK/KUP* family can be divided into four clusters, each of which includes genes that are localised in different subcellular membranes (Gupta et al., 2008). A study on *cis*-elements, which play an important role in gene regulation, showed that the upstream region of most *OsHAK* genes contains Ca^{2+}-responsive *cis*-elements. As these may be associated with the key role of Ca^{2+}, as a second messenger in response to environmental stimuli, Ca^{2+} is able, therefore, both to activate and deactivate the *OsHAK* genes, by regulating its *cis*-elements (Gupta et al., 2008).

1.7.2.1 Expression pattern of the *KT/HAK/KUP* family in rice plants

A study on the expression of *OsHAK1* and *OsHAK7* in the shoots and roots of rice plants under high- and low-K^+ conditions, showed that although *OsHAK1* expression was found in the whole plant, with the highest expression identified in the root, *OsHAK7* was expressed only in the shoots and root. This suggested that OsHAK1 belongs to the high-affinity transporters in rice roots (Bañuelos et al., 2002). An *in situ* hybridisation analysis showed that *OsHAK1* was mainly expressed in the root tips and, specifically, in their meristematic regions. The OsHAK1 mRNA

signal indicated that *OsHAK1* expressed in the epidermal cells and vascular cells, while, in the shoots, it was detected in the apical meristem, and in the cells on the inner side of the vascular bundle of leaf sheaths, as well as at the conjunction of the root and shoot, and in the stem (Chen et al., 2015). Another study which investigated the expression of 26 *OsHAK* genes in the roots of rice seedlings, detected 14 genes that were involved in either K^+ uptake or some other related function (Gupta et al., 2008). In Arabidopsis, evidence of *OsHAK5* expression was found in the epidermis of the main and lateral roots, and, to lesser extent, in the vasculature of the main roots (Gierth et al., 2005). In contrast, overexpression of *OsHAK5* in rice plants increased the net K^+ influx rate by 2.6-fold under a 0.1 mM K^+ condition, suggesting OsHAK5 plays a role in root K^+ acquisition under a K^+-deficient condition. Moreover, the fact that a strong expression of *OsHAK5* was identified in the xylem parenchyma and phloem of root vascular tissue, particularly under a K^+-deficient condition, suggested that OsHAK5 may be involved in the distribution of K^+ transport between root and shoot (Yang et al., 2014).

1.7.2.2 *HAK* genes mediate high-affinity K^+ uptake in plant roots

Several studies indicated that HvHAK1 functions as a high–affinity K^+ transporter in Arabidopsis plants (Mangano et al., 2008; Santa-María et al., 1997). When the plants were grown in media containing 1 mM KCl, the overexpression of *HvHAK1* resulted in a slightly higher Rb^+ uptake, which was more significant in plants under the K^+-limiting condition than in the control plants (Fulgenzi et al., 2008). When the effect that knocking out *OsHAK1* had on of rice plants grown under two K^+ levels, 0.1 and 1 mM K^+, was studied by Chen et al (2015), results showed that the K^+ uptake rate in the roots of *Oshak1* mutants was about 50%, and 70%, respectively, with the total K^+ uptake per plant being 15-20%, and 30-35%, respectively, and the K^+ acquisition, 15%, and 35%, respectively, compared to wild type plants. Thus, while the *Oshak1* mutant plants showed significant growth impairment under both conditions, this was more pronounced under the low K^+ condition (Chen et al., 2015). Moreover, knocking out *Oshak1* limited cell expansion,

resulting in smaller mutant plants (Chen et al., 2015). Another study highlighted the key role played by *AtHAK5* both in seedling establishment and plant growth, under a low-potassium condition (Pyo et al., 2010). *Athak5* mutant plants showed lower root and shoot K^+ content, compared to the wild type, when Arabidopsis plants were grown under K^+-deficient conditions (Gierth et al., 2005; Nieves-Cordones et al., 2010; Pyo et al., 2010). It was consistently found that under a K^+ deficient condition, overexpression of *AtHAK5* enhanced the growth of Arabidopsis plants, by causing them to produce a greater number and density of lateral roots, and a higher dry weight of shoots and roots compared to the wild type (Adams et al., 2014). A comparison study of the growth of overexpressed *OsHAK5* and *Oshak5* mutant plants and their wild type showed that, when the plants were grown in low-potassium conditions, the expression of *OsHAK5* was rapidly up-regulated in the plasma membrane of the epidermal cells of the rice root, resulting in increased acquisition. However, the *Oshak5* mutant plants accumulated only 60% of the K^+ accumulated by their wild type (Yang et al., 2014). In contrast, when grown in a medium containing 1 mM K^+, the overexpressed *OsHAK5* plants did not display a significant difference in their growth, compared to the wild type. The suggestion from this is that OsHAK5 was involved in K^+ acquisition in the rice plants under the low-potassium condition (Yang et al., 2014).

1.7.2.3 The function of *HAK* genes in K^+ uptake under salt stress conditions

According to Chen et al. (2015), *OsHAK1* expression was regulated differently by salt stress, depending on the external K^+ concentration. For example, under a normal K^+ condition, the addition of Na^+ up-regulated *OsHAK1* expression, while, under a low-potassium condition, it was reduced. This resulted in a lower K^+ uptake rate in the wild type, while the *Oshak1* mutant plants uptake was completely blocked. The study concluded that all high-affinity K^+ transport systems in rice plants, including OsHAK1, OsHAK5 and OsAKT1, are salt-sensitive (Chen et al., 2015). Results of a study by Fulgenzi et al. (2008) consistently showed that the *HvHAK1* transcript was significantly up-regulated by 100 mM NaCl under a 1 mM K^+ condition, resulting in a significant

K^+ uptake after 6 h of salt induction. In Arabidopsis plants grown under a low-potassium condition, while the expression of *AtHAK5* was up-regulated, accompanied by a high K^+ uptake rate by the root, it was significantly reduced by the addition of Na^+ to the medium (Nieves-Cordones et al., 2010). One possible explanation for this is that the Na^+ induced depolarisation of the membrane potential that, in turn, regulated the activity of the *AtHAK5* gene (Nieves-Cordones et al., 2008). However, the differences in the Na^+ concentration between the overexpressed *HAK5* lines and the wild type was insignificant, regardless of whether they were under control, K^+-deficiency, or salt stress conditions. Furthermore, since no morphological phenotype was found in the overexpressed lines under salt stress, this suggested that *AtHAK5* is not involved in Na^+ uptake in Arabidopsis plants (Adams et al., 2014; Nieves-Cordones et al., 2010). In contrast, Yang et al. (2014) reported that, even under normal K^+ conditions, the *OsHAK5* expression was vastly up-regulated by salinity. Thus, the overexpressed *OsHAK5* mutant plants were salt-tolerant, and the *Oshak5* mutant plants salt-sensitive, compared to the wild type, under a 100 mM NaCl condition. This suggests that OsHAK5 improves rice salt tolerance by enhancing the shoot K^+: Na^+ ratio. Moreover, the presence of OsHAK5 was detected in mesophyll cells and phloem tissue (Yang et al., 2014). In general, the improvement of plant-K^+ acquisition and growth under salt conditions by enhancing the transcript levels of genes that encode the high-affinity potassium transporters is an important target for plant breeders (Nieves-Cordones et al., 2010).

1.7.2.4 The role of *HAK* genes in K^+ transport

Significant expression levels of *OsHAK1* and *OsHAK5* were found in the xylem parenchyma and phloem of root vascular tissue, suggesting a role for OsHAK1 and OsHAK5 in K^+ transport from root to aerial parts (Chen et al., 2015; Yang et al., 2014). The root K^+ content of overexpressed *OsHAK5* plants, compared to the wild type, was 30%, and 60% lower, under 0.3 mM K^+, and K-free conditions, respectively. While, under a normal K^+ condition (1 mM), the overexpression of *OsHAK5* did not contribute to the transport of K^+ from root to shoot (Yang et

al., 2014), under the low-potassium condition, OsHAK5 and OsHAK1 both played a significant role in the xylem loading (Chen et al., 2015; Yang et al., 2014), with the K^+ content of the xylem sap being higher by 20-25% in the overexpressed line, and lower in the *oshak5* mutant line, compared to the wild type. This leads to the conclusion that, under a normal condition, other K^+ channels and K^+ diffusion via apoplast may play an essential role in K^+ transport from root to shoot, while under a low-K^+ condition, OsHAK5 mediates K^+ transport (Yang et al., 2014).

1.7.3 The HKT Family of transporters

Salinity tolerance is strongly related to controlled Na^+ uptake, long-distance transport and accumulation in photosynthetic plant tissue (Maathuis, 2014; Tester and Devenport, 2003). Although a number of Na^+ transporters have been shown to contribute substantially to Na^+ tolerance in plants, it is the HKT (High-affinity K^+ Transporter) membrane transporter that plays the crucial role in plant salt tolerance. This prominence is due to its permeability to both K^+ and Na^+, or to Na^+ alone, both in monocotyledons such as rice, and dicotyledons such as Arabidopsis. This function makes HKT transporters an important target for research into breeding plants towards salt tolerance.

There follows, now, an overview of the structure and physiological function of HKT in plants, with a special focus on rice.

1.7.3.1 HKT structure in plants

It has been reported that the families Trk/Ktr/HKT and HAK/Kup/KT are found only in plant cells where they are specifically involved in the transport of K^+ in bacteria, fungi, and plants, and that (Corratgé-Faillie et al., 2010). Trk/Ktr/HKT transporters (Trk in fungi, Trk, and Ktr in bacteria, and HKT in plants) are purported to play a key role in ion homeostasis through K^+ and Na^+ uptake, osmotic adjustment, and Na^+ recirculation from shoot to root (Corratgé-Faillie et al., 2010).

A model of the protein structure of the Trk/Ktr/HKT transporters was proposed based on the crystal structure of the KcsA bacterial channel in *Streptomyces lividans*, and on multiple-sequence alignments of conserved residues (Durell and Guy, 1999; Durell et al., 1999). This model, which predicts HKT proteins, appears to contain four sequential M1-P-M2 motifs (MPM) of which each has two fully transmembrane helices (M1 and M2) plus an intervening hairpin segment (P) that determines the ion selectivity (Durell et al., 1999). This structure has been confirmed by an experiment that examined the topology of the AtHKT1;1, a protein purported to mediate the Na^+ inwardly in *Xenopus laevis* oocytes, and K^+ uptake in *Escherichia coli*. The results showed that AtHKT1;1 contains eight transmembrane-spanning segments, and also NH_2- and COOH- terminals that face the cytosol side. Thus, the topology of AtHKT1;1 contains four proposed membrane-pore-membrane motifs (MPM_A, MPM_B, MPM_C, and MPM_D) (Kato et al., 2001) (Fig. 1.4). Amino acid (G), present in the first pore loop, is the most highly conserved residue of the channels, and symportes sequences. Thus, it has been proposed as the determining factor for ion selectivity in these proteins (Durell and Guy, 1999).

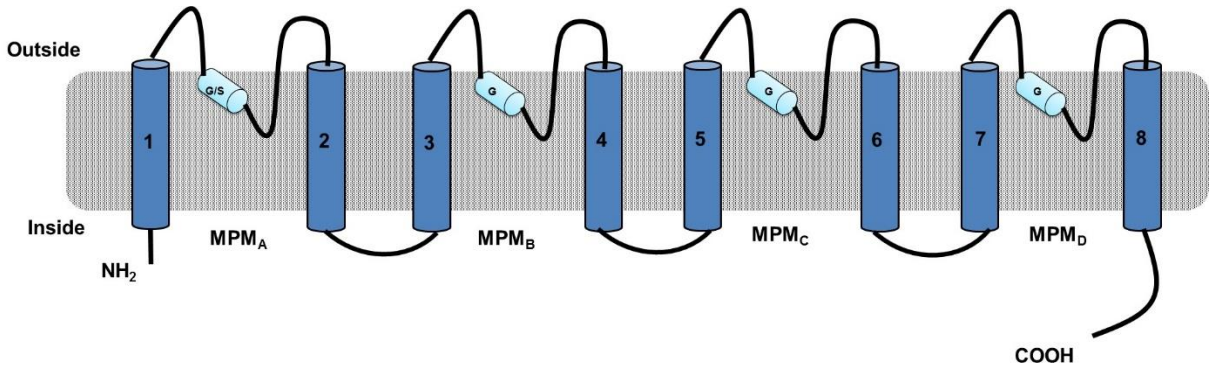

Figure 1. 2 The structure of HKT transporters. The model consists of four membrane-pore-membrane (MPM) motifs. Subfamily one of Na^+ selective transporters retains a serine (S) residue in the first pore domain, whereas sub-family two shows K^+ permeability due to glycine (G) residue in all pore domains, with the exception of OsHKT2;1 which, similar to sub-family one, retains (S) residue in the first pore domain, and shows strong Na^+ selectivity (Corratgé-Faillie et al., 2010; Durell and Guy, 1999; Durell et al., 1999).

1.7.3.2 The HKT sub-families

HKT transporters have been classified by Platten et al. (2006) into two sub-families, based on their gene structure and whether they have a G and/or S in the pore loop with some exceptions. Subfamily 1 is a Na^+ uniporter due to the presence of amino acid serine (S) in the first pore domain (Mäser et al., 2002b), while the presence of amino acid glycine (G) in the first pore domain enables the Subfamily 2 transporters to select either Na^+ and/ or K^+, depending on the Na^+ concentration in the soil solution (Almeida et al., 2013). In reality, this classification system is not as simple as this as there are a number of exceptions. For example, the exceptions for Subfamily 1 are Eucalyptus HKTs EcHKT1;1 and EcHKT1;2, which can transport both Na^+ and K^+ (Liu et al., 2001). In Subfamily 2, the exception is OsHKT2;1 since it can transport K^+ despite the presence of amino acid (S) in the first pore domain (Golldack et al., 2002), and OsHKT2;4, which is permeable both to Ca^{2+} and Mg^{2+} (Horie et al., 2011). The Subfamily 1 includes all the *HKT* genes of the Dicot species. In contrast, monocot rice possesses nine *HKT* genes, which are divided between the two sub-families (Platten et al., 2006).

1.7.3.3 The *HKT* gene family in rice

In the rice genome, a total of nine *HKT* genes, belonging to both Subfamilies 1 and 2, have been identified (*OsHKT1;1-OsHKT1;5* and *OsHKT2;1-OsHKT2;4*) (Platten et al., 2006) Table 1). Earlier, *OsHKT2;1* and *OsHKT2;4* members of the HKT family were identified in japonica rice, cv Nipponbare, and indica rice, cv Pokkali (Horie et al., 2001). Another eight genes, including *OsHKT2;1*, *OsHKT2;3*, *OsHKT2;4*, *OsHKT1;1*, *OsHKT1;5*, were identified in rice, cv Nipponbare by carrying out systemic BLAST research on public databases and in the Monsanto Rice Genome Sequences, using the amino acids sequences of wheat HKT1, Arabidopsis HKT1, and rice HKT2;1 and HKT2;2 (Garciadeblas et al., 2003).

The *HKT* genes of rice can be classified into two groups, based on the length of their gene sequence: those in Group 1 (*OsHKT2;1*, *OsHKT2;3*, *OsHKT2;4*, *OsHKT1;1*, *OsHKT1;2*,

OsHKT1;3, OsHKT1;4) have two introns of moderate length with reference to the length of the genes; and those in Group 2 (*OsHKT1;4* and *OsHKT1;5)* have larger introns. Furthermore, all rice OsHKT transporters could be classified as KcsA (pH-gated potassium channel)- related transporters (Garciadeblas et al., 2003)

When studied using phylogenetic analysis, these rice HKT transporters were found to be very divergent. OsHKT1;1 and OsHKT1;2 kept 60% of their identity, while all the other genes sequences only kept between 40 and 50% of theirs. The exceptions to this were the pairs formulated by OsHKT2;3 and OsHKT2;4, and by OsHKT2;1 and Po- OsHKT2;2, which kept 93%, and 91% of their identity, respectively (Garciadeblas et al., 2003).

1.7.3.4 Expression patterns of *HKT* genes in rice plants

OsHKT1;1 and *OsHKT1;3* are expressed in peripheral layers (the epidermis, exodermis, and cortex) as well as in vascular tissue (mainly in the phloem, but also in the stele) of roots. In mature leaves, the transcripts of both genes were detected in bulliform cells, especially in the vacuolated cell of the adaxial epidermis, while a low expression of *OsHKT1;3* was found in mesophyll (Jabnoune et al., 2009).

The transcript of *OsHKT1;2* could not be detected in rice roots, and it showed slight differences in the leaves under the effects of different Na^+, and K^+ treatments (Wu et al., 2008). This same gene was also reported to be non-functional in cv. Nipponbare (Garciadeblas et al., 2003).

OsHKT2;1 is expressed in the root in the epidermis, exodermis, cortex, and stele, mainly in the phloem. In the leaves, its expression was detected in bulliform cells (Golldack et al., 2002; Jabnoune et al., 2009). Under normal conditions, *OsHKT2;2* was mainly expressed in the epidermis and vascular cylinder of the root, with a lower expression evident in the cortex. In the leaf tissue, *OsHKT2;2* expression was found mainly in the mesophyll cells, while under salt stress

a higher expression level of it was detected in the phloem and in the area between the phloem and the mesophyll cells (Kader et al., 2006).

It has been found that OsHKT2;3 and OsHKT2;4 share approximately 93% of their identity at the amino acid sequence level, and are the only Subfamily 2 transporters that conserve (Gly) residues at the four P-loop filter positions in Nipponbare, making both of them the most closely related of all the identified HKT transporters (Horie et al., 2011). *OsHKT2;3* could not be cloned from the root, but was successfully cloned from the shoot (Garciadeblas et al., 2003), suggesting that the *OsHKT2;3* is only marginally expressed in the root in comparison to the shoot.

OsHKT2;4 was found to be expressed in spikelets, leaves, leaf-sheathes, internodes, nodes, the bases of stems, roots and lateral roots, but its expression was less likely to be detected in mesophyll cells (Lan et al., 2010) Table (1.1).

Table 1. 1 Table listing the nine HKT transporters that have been found in rice plants, as well as their expressions in those plants (Almeida et al., 2013).

Transporter	Expression in planta	Reference
OsHKT1;1	In root: at peripheral layers and vascular tissues. In leaves: at bulliform celles	(Jabnoune et al., 2009)
OsHKT1;2	In leaves and not detected in root	(Wu et al., 2008)
OsHKT1;3	In root: at peripheral layers and vascular tissues. In leaves: at bulliform celles	(Jabnoune et al., 2009)
OsHKT1;4	in leaf sheath tissue	(Cotsaftis et al., 2012)
OsHKT1;5	in xylem parenchyma cells of the root and leaves	(Cotsaftis et al., 2012)
OsHKT2;1	In root (epidermis, exodermis, cortex, stele). In leaves: bulliform cells	(Golldack et al., 2002)
OsHKT2;2	In root: cortex, epidermis, vascular cylinder. In leaves: mainly mesophyll	(Kader et al., 2006)
OsHKT2;3	Mainly in the shoot and marginally in the root of plant	(Garciadeblas et al., 2003)
OsHKT2;4	Spikelets, leaves, leaf sheathes, internodes, nodes, root, mesophyll cells	(Lan et al., 2010)

1.7.3.5 The role of *HKT* genes of Subfamily 1 in Na^+ detoxification

Although OsHKT1;1 to OsHKT1;5 of the Sub-family 1 are reported only to be permeable to Na^+ due to the earlier-mentioned presence of amino acid serine (S) residue in the first pore domain (PD), they differ strongly in terms of their affinity for Na^+ rectification, and their sensitivity to external K^+ (Jabnoune et al., 2009; Ren et al., 2005). OsHKT1;1 was proven to mediate low-affinity Na^+ uptake. This uptake was also shown to be competitively inhibited by K^+ and Ba^+. Thus, OsHKT1;1 was sensitive to K^+ and Ba^+ concentrations. Additionally, the capacity of OsHKT1;1 to transport K^+ was lower than its capacity to transport Na^+ (Garciadeblas et al., 2003).

The expression of *AtHKT1;1* in the outer cells both of Arabidopsis and rice roots improved their salinity tolerance by minimising the concentration of Na^+ in their shoots. In rice expressing *AtHKT1;1*, the up-regulation of *OsHKT1;5* expression, as well as an increase in the Na^+ sequestration capacity of the cortical cells, due to increased expression of rice vacuolar H^+ translocating pyro-phosphatases, are the two main explanations for this result (Plett et al., 2010).

In the D and A genomes of salt sensitive and salt tolerant varieties of wheat, the expression of the *TaHKT1;5-D* and *TaHKT1;5-A* alleles was studied under different salt concentrations. The results showed that in response to salinity, *HKT1;5* expression was detected earlier in the leaves than the roots. Genotypically, *HKT1;5* expression appeared earlier in salt-tolerant varieties than in salt sensitive ones. *TaHKT1;5-D* had a higher expression than *TaHKT1;5-A*, a finding supported by Byrt et al. (2014). TaHKT1;5-D was found to have a significant role in minimising the transport of Na^+ from the root to the leaves in bread wheat, by retrieving Na^+ from the xylem vessels in the roots. Of particular note are the allelic variants in OsHKT1;5; these were found to play such an important role in rice salt tolerance that they may hold the key to rice-breeding (Negrao et al., 2013). These results suggest that Na^+ exclusion is the main salinity tolerance mechanism in wheat, and that HKT1;5 plays a significant role in activating this mechanism by excluding Na^+ from

leaves while controlling xylem loading in the roots, thereby maintaining a high K^+:Na^+ ratio in the leaves (Zamani Babgohari et al., 2013).

Under saline conditions, it appears that OsHKT1;4 is a sheath-specific transporter that plays a key role by controlling the sheath-to-blade transfer of Na^+ in rice (Cotsaftis et al., 2012). Interestingly, *OsHKT1;4* transcript was higher in the young leaves of rice, cv.Pokkali, while being lower in cv. Nipponbare. Therefore, the former performed better than the latter under salt stress by (a) minimising the expression of *OsHKT1;4* in older sheaths, in order to direct and store Na^+ in senesing photosynthetic tissues, as well as by (b) maximising *OsHKT1;4* expression in younger sheaths to preserve them, and increase plant survival under salt stress (Cotsaftis et al., 2012). OsHKT1;2 and OsHKT1;3 have been less widely studied. OsHKT1;3, which can be a low-affinity, slightly- or strongly-rectifying transporter, has been shown to transport only Na^+ (Jabnoune et al., 2009).

1.7.3.6 The role of *OsHKT* Subfamily 2 genes in K^+ and Na^+ transport

It has been suggested that OsHKT2;1 and OsHKT2;4 encode K^+/ Na^+ co-transporters. This is based on the presence of amino acid glycine in the first pore domain of their amino acid sequence. Contrary to expectations, OsHKT2;1 has amino acid (S) in the first pore domain, making its transport capacity of Na^+ similar to that of the Sub-family1 of *HKT* genes.

OsHKT2;1 has been widely studied to determine its functional roles. A characterisation study of the effects of alkali cations on the function and expression of *OsHKT2;1* in the salt sensitive rice IR29 and the salt tolerant Pokkali, showed that the concentration of K^+ and Na^+ in the leaves was not significantly different when the plan was grown at 0, 0.1, and 4 mM of K^+. Both lines accumulated a high concentration of Na^+ in their leaves, but not in their roots, when the plants were exposed to salinity. In the presence of K^+, Pokkali accumulated 30% less Na^+ than IR29, after 72 h of salt treatment. In the absence of K^+, the Na^+ concentration in the leaf-tissue of both

lines was similar. However, the Na^+ content was affected by the concentration of the K^+ in the nutrient solution. In response to alkali-salt stress, *OsHKT2;1* transcription was down-regulated in the roots and leaves of Pokkali, lowering the Na^+ (Li^+ and Rb^+) concentration in the plant, whereas in IR29 it either remained unchanged, or down-regulated to a lesser extent. These results suggest that the regulation of *OsHKT2;1* expression by salt stress is an important mechanism in salt tolerance, where OsHKT2;1 is involved in xylem loading, the long-distance transport of alkali cations from roots to shoots, and in leaf phloem loading (Golldack et al., 2002). Consistent with these results, Horie et al. (2007) and Yao et al. (2010) reported that OsHKT2;1 mediated Na^+ uptake as well as the uptake of a small amount of Rb^+. Furthermore, in rice roots, Na^+ uptake was inhibited by the inhibition or down-regulation of the OsHKT2;1 transporter in the presence of external K^+ and Ca^{2+}. Recently, a study on the effects of the expression of *OsHKT2;1* on Na^+ accumulation in rice plants, revealed that a higher *OsHKT2;1* expression enhanced Na^+ accumulation in shoots under low external K^+, while under sufficient external K^+, it resulted in more Na^+ being accumulated in the roots but not in the shoots. These results point to the expression of this particular gene as being an important l factor in Na^+ homeostasis in rice plants (Miyamoto et al., 2015).

Although OsHKT2;1 and OsHKT2;2 share 91% of their sequence identity and also have similar hydrophobic profiles, OsHKT2;2 differs from OsHKT2;1 in its K^+ transport properties. An investigation of *X. laevis* oocytes that expresses these transporters, using the electrophysiological technique, demonstrated that OsHKT2;2 exhibits an intriguing behaviour with regard to its ion specificity. OsHKT2;1 did not mediate K^+ influx in a high K^+ solution and in the absence of Na^+, while in the absence of this external K^+, a large inward current was elicited by external Na^+. Thus, OsHKT2;2 was predicted to be a Na^+/K^+ coupled transporter (Horie et al., 2001).

An expression analysis of *OsHKT2;2*, both in salt tolerant Pokalli and salt sensitive BRRI Dhan29 under salt stress, showed that although *OsHKT2;2* transcripts remained unaffected in

BRRI Dhan29, they immediately increased in Pokkali shoot tissue, by 15-fold after 1h of salt stress and by 158% after 72 h, compared to the control. In the root, up-regulation of *OsHKT2;2* was detected in the root of Pokkali after 72 h of salt treatment, but was not as high as in the shoot tissue. Meanwhile, down-regulation of *OsHKT2;2* was observed in the root of BRRI Dhan29 after 24 h of salt stress. The induction of OsHKT2;2 in the epidermis, exodermis, and xylem tissue of roots indicates its role in K^+ uptake, and transport via xylem. Likewise, its induction in the mesophyll cells and the transition point from the phloem to mesophyll cell may be an indication of its role in K^+ recirculation within the mesophyll cells, via the phloem. Thus, OsHKT2;2 may confer salt tolerance in cv. Pokalli by enhancing the K^+/Na^+ ratio in its leaf tissue (Kader et al., 2006).

The physiological roles of OsHKT2;3 are yet to be confirmed. However, since OsHKT2;3 shares 93% of its amino acid sequence with OsHKT2;4, it is believed to also be a Na^+ and K^+ co-transporter (Horie et al., 2011). In Nipponbare, under various Na^+ concentrations, OsHKT2;3 could not be detected in the root, and caused only a small change in the Na^+ concentration in the leaves (Wu et al., 2008). When *OsHKT2;3* expressed in the K^+ uptake-deficient CY162 and the Na^+ sensitive G19 strain of *Saccharomyces cerevisiae* under 0,1 mM K^+, and 200 mM NaCl, respectively, no growth difference was detected in the mutant yeast cells of either, compared to their vector-harboring control cells (Horie et al., 2011).

A kinetic analysis of OsHKT2;4 demonstrated that it mediated low-affinity Na^+ uptake, and that its capacity for K^+ transport is much lower than that for for Na^+ transport (Garciadeblas et al., 2003). Another study tested the physiological role of OsHKT2;4, by performing an electrophysiological analysis of OsHKT2;4 in *Xenopus* oocytes. The results showed that oocytes in which *OsHKT2;4* expressed, generated current that appeared to contain two components with different kinetics. Furthermore, to evaluate the nature of OsHKT2;4, several cations were used to determine its ionic contribution to this current. When Ca^{2+} was added, the OsHKT2;4 current was

significantly larger than that generated by other cations. Thus, OsHKT2;4 may function as a Ca^{2+} permeable channel. Moreover, since Ca^{2+} determined the kinetics of the OsHKT2;4 current in oocytes, the presence of Ca^{2+} may enhance the influx of K^+, Na^+ and Mg^{2+} into plant cells (Lan et al., 2010). Such an hypothesis has been refuted by the results of another experiment aimed at studying the ion selectivity of OsHKT2;4 in *Xenopus* oocytes. The results showed that while OsHKT2;4 permeability was not detected even at a very low concentration of external K^+, it did show a higher permeability to K^+ than Na^+, with a permeability sequence as follows: $K^+ > Rb^+ \approx Cs^+ > Na^+ \approx Li^+ \approx NH4^+$ (Sassi et al., 2012). When other cations were tested, OsHKT2;4 proved to be permeable to many of the monovalent cations, such as NH_4^+, Li^+, Na^+ and K^+, as well as to the divalent cations Zn^{2+}, Mn^{2+}, Cu^{2+}, Fe^{2+} and Cd^{2+}. On the basis of results obtained using K^+ and Ca^{2+} channel blockers, HKT2;4 may have at least two binding sites: one for small cations, such as Na^+ and K^+, and another for larger cations, such as Ca^{2+} (Lan et al., 2010). When *OsHKT2;4* expressed in the K^+ uptake-deficient CY162 and the Na^+ sensitive G19 strains of *S. Cerevisiae*, the growth of both was improved, under 0,1 mM K^+, and 200 mM NaCl, respectively. The results of a study on the ion selectivity of OsHKT2;4, suggest that OsHKT2;4 functions as a K^+ permeable transporter/channel, with a smaller permeability to Ca^{2+} and Mg^{2+}, and the latter function is competitively inhibited by K^+ (Horie et al., 2011). The physiological role of some of the HKT and HAK transporters is summarised in (Fig. 1.3).

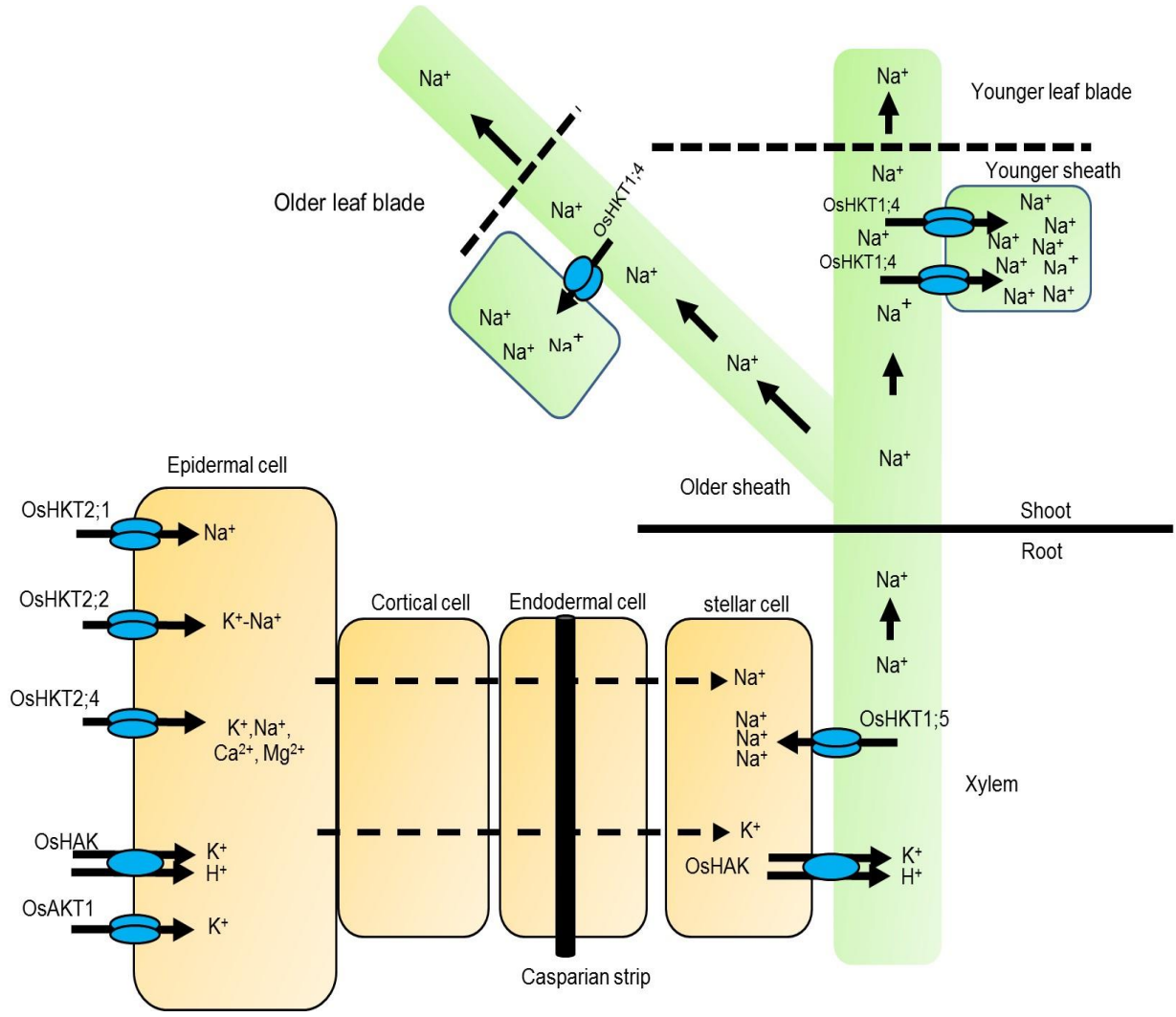

Figure 1. 3 Model showing the physiological role of some of the HKT and HAK transporters, and the OsAKT1 channel in radial and long-distance Na^+ transport from the root to the shoot in rice plants. OsHKT2;1 may have a function as a transporter of Na^+ to root tissue, while in the same tissue, OsHKT2;2 functions both as a Na^+ and K^+ transporter, and OsHKT2;4 as a transporter of Na^+, K^+, Ca^{2+}, and Mg^{2+}. *OsHKT1;5 was* expressed in xylem parenchyma, unloading Na^+ from the xylem back to the root tissue, and thereby controlling Na^+ accumulation in the shoot. In the shoot, a higher expression level of *OsHKT1;4* in younger sheaths causes more Na^+ to accumulate thereby minimising the amount of Na^+ going to photosynthetic tissue, while its lower expression in older sheaths results in Na^+ being stored in the senescing leaf blades without affecting plant growth (Almeida et al., 2014; Cotsaftis et al., 2012; Kader et al., 2006; Su et al., 2015). *OsAKT1* is expressed in the epidermis, exodermis and endodermis of rice roots, where it functions in K^+ uptake and translocation (Golldack et al., 2003). Both *OsHAK1* and *OsHAK5* are strongly expressed in the epidermis of the main and lateral roots, as well as in the xylem parenchyma and

phloem of root vascular tissue, especially under K^+-deficient condition (Chen et al., 2015; Yang et al., 2014)

1.8 Abiotic stress signalling pathways in plants

A knowledge of the physiological and metabolic mechanisms by which plants perceive biotic and abiotic stresses, and the subsequent signal transduction that activates their adaptive responses, is vital to improving the salt, drought, heat, and cold tolerance of plants. Zhu (2002a) proposed three categories of salt and drought signaling: ionic and osmotic signaling, detoxification signaling, and signaling associated with the control of plant growth. Various components of signaling transduction have been identified, including the creation of a network of protein-protein reactions and ion channels, the production of reactive oxygen species (ROS), and the accumulation of hormones such as abscisic acid (ABA), and kinase cascade and Ca^{2+} as second messengers (Demiral et al., 2011). These findings thus raise the question of how Ca^{2+} and ROS transduce different signaling pathways in response to abiotic stress.

1.8.1 Ca^{2+} signature

Calcium and the reactive oxygen species play a vital role as second messengers in plant responses to biotic and abiotic stress. Under normal conditions, the cytosolic concentration of calcium $[Ca^{2+}]_{cyt}$ is maintained at a nanomolar level, generally in the range of 100-200 nM (Bush, 1995), whereas it is in the millimolar range (1-10 mM) in extracellular and intracellular Ca^{2+} stores. Under adverse environmental conditions, this cytosolic concentration can be higher, up to millimolar level. Plants not only store the excess Ca^{2+}, either in the apoplast or in the lumen intracellular organelles, in order to avoid Ca^{2+} toxicity, but also use it as a signal transductor, in response to adverse environmental conditions (Kader and Lindberg, 2010; Perochon et al., 2011; Reddy and Reddy, 2004). In response to environmental stress, the increase in the cytosolic Ca^{2+} concentration (so called "Ca^{2+} signature") is a result of the activation of Ca^{2+} channels, pumps, and transporters located in the plasma membrane or in other organelle membranes, resulting in the

downstream response of targeted genes (Tuteja and Mahajan, 2007). The peak, magnitude, frequency and duration of the Ca^{2+} signature play a vital role in encoding specific information for plants under various conditions (Whalley and Knight, 2013). The Ca^{2+} signature must be either of low amplitude or transient. The latter can be single (spike), double (biphasic), or multiple (oscillations) (Tuteja and Mahajan, 2007). The results of a study on the effect of abiotic stress on the calcium signature of two ecotypes of Arabidopsis, Col-0 and C24, showed that 200 mM NaCl caused a peak with a magnitude of 800-855 nM, while under osmotic stress generated by 400 mM of sorbitol, the peak magnitude ranged between 73 and 750 nM, depending on the particular ecotype of Arabidopsis (Schmöckel et al., 2015). Interestingly, these results showed that in the case of the Col-0 ecotype, the double (biphasic) Ca^{2+} signatures occurred between 30-120 seconds after salt treatment and lasted approximately 30s, while the C24 had only one peak (spike). This suggested that the salt signaling pathway is triggered by NaCl, and initiates a signal cascade causing Ca^{2+} influx into the cytosol to create a Ca^{2+} signature (Schmöckel et al., 2015).

1.8.2 ROS and intracellular Ca^{2+} signature

ROS are not always toxic molecules that need to be detoxified, for the signals resulting from the spatial and temporal expression of ROS waves are required by plants for growth, development, PCD, and biotic and abiotic responses (Gechev et al., 2006). It has been reported that an increase in both cytosolic Ca^{2+} and ROS production is important for the maintenance of an adequate K^+:Na^+ ratio in plants under salt stress (Ma et al., 2012a). Moreover, several studies have provided evidence of an interaction between Ca^{2+} and ROS. ROS-activated Ca^{2+} and K^+ transporters in the plasma membrane of Arabidopsis root protoplast, mediate both Ca^{2+} influx and K^+ efflux (Demidchik et al., 2003). In Arabidopsis guard cells, hyperpolarisation of the plasma membrane by ROS, increased the cytosolic Ca^{2+} concentration, suggesting that under drought stress, ABA induced H_2O_2 production, which, in turn, activated Ca^{2+} channels, the process that is vital for ABA-induced stomatal closure and, ultimately, plant drought tolerance (Pei et al., 2000).

In Arabidopsis, the genes of the double mutants, *AtrbohD* and *Atrbohf*, inhibited ROS generation, resulting in a higher Na^+ and lower K^+ content, and therefore, a higher Na^+: K^+ ratio, compared to the wild type. Moreover, in the double mutant plants, exogenous H_2O_2 can partially mitigate the adverse effects of salinity on the Na^+: K^+ ratio (Ma et al., 2012a). Respiratory burst oxidase homologs (Rbohs) are synergistically activated by Ca^{2+}, binding to the EF-hand motifs and, also, by phosphorylation (Ogasawara et al., 2008). It has been reported that CIPK26 phosphorylates RBOHF *in vitro*, enhancing ROS production. This suggests a relationship between CBL-CIPK-mediated Ca^{2+} and ROS signaling (Drerup et al., 2013).

1.8.3 Ca^{2+} binding proteins

Plants have evolved a specific set of proteins that bind Ca^{2+}, with or without helix-loop-helix EF-hand motifs. Three major categories of EF-hand proteins in plants are the calmodulins (CaMs) and CaM-like proteins (CMLs); Ca^{2+}-dependent protein kinases (CDPKs); and calcineurin B-like proteins (CBLs) (DeFalco et al., 2010). CaMs are found in all eukaryotes, while CMLs and CDPKs are restricted to plants and some protists (DeFalco et al., 2010). Some of these Ca^{2+} binding proteins, such as CPDKs, when bound to Ca^{2+}, can immediately regulate downstream proteins (Schulz et al., 2013), while others, the CMLs and CBLs, bind to other proteins to affect their downstream target.

CBL-interacting protein kinases (CIPKs) are a unique family of serine/threonine protein kinases, present in plants. There is evidence from various studies that biotic and abiotic stresses, such as salinity, drought, cold and high pH, induce different expression patterns and sub-cellular localization of CBL sensors and CIPKs kinases (Kolukisaoglu et al., 2004; Li et al., 2009; Manik et al., 2015). This role of CIPKs proteins in plant abiotic stress is addressed in the following sections.

1.8.3.1 The functional role of CIPKs in sodium signaling

A considerable number of salt-responsive genes are involved in the sodium signaling pathway, where their role is to minimise the toxicity of the Na^+ ion while maintaining ion homeostasis. The salt-overly-sensitive (SOS) pathway is the classic example of a CBL/CIPKs interaction. The SOS3/CBL4 activates CIPK-type kinase SOS2/CIPK24 activity in a Ca^{2+}-dependent manner. The SOS3/SOS2 complex, in turn, phosphorylates and activates the Na^+/H^+ anti-porter, SOS1. It is this activated SOS1 that then enhances salt detoxification, through Na^+ extrusion into the extracellular medium (Halfter et al., 2000). The OsCLB4-OsCIPK24 complex have been shown to activate OsSOS1 in yeast cells, thereby reducing their net cellular Na^+ content. These results also confirm the role played by the SOS pathway in salt tolerance in rice (Martínez-Atienza et al., 2007). Another calcium sensor, SOS3, was found to activate the kinase SOS2 that up-regulates the plasma membrane Na^+/H^+ antiporter, SOS1, in plants. In addition, SOS3 was found to act mainly in roots under salt stress, while *CBL10* is the only one expressed strongly in shoots; its expression in roots was very weak (Quan et al., 2007). A study by Chen et al. (2013) found that overexpression of the *CIPK6* gene in response to salt, osmotic stress and ABA enhanced plant salt stress, and increased plant sensitivity to ABA. A total of 24 *TaCBLs* and 79 *TaCIPKs* were identified in the genome of bread wheat (*Triticum aestivum L*) (Sun et al., 2015). The expression of *TaCIPK24*, the orthologous of Arabidopsis SOS2 was up-regulated by salinity in the root and leaf tissues of wheat. Moreover, the overexpression of *TaCIPK24* improved plant salt tolerance by enhancing the enzymatic antioxidant system, including catalase (CAT), peroxidase (POD), and superoxide dismutase (SOD), and increasing Na^+ efflux in transgenic plants, compared to the wild type (Sun et al., 2015). *TaCIPK29* was identified as a new member of the CIPK family in wheat, and was shown to be involved in ion and ROS homeostasis. Its transcript increased in response to NaCl, cold, and ABA treatments. Moreover, in transgenic tobacco, the overexpression of *TaCIPK29* resulted in a higher germination rate, longer root, a higher K^+: Na^+ ratio, and Ca^{2+}

content, lower H_2O_2 levels, an enhanced enzymatic antioxidant system, better growth rate, and ultimately improved plant salt tolerance (Deng et al., 2013). *AtCIPK21* showed a ubiquitous expression in plant tissues, which was positively regulated by the different stimuli. Under salt conditions, CBL2 and CBL3 sensors were found to interact physically with CIPK21 on the tonoplast (Pandey et al., 2015). This same study also noted that mutant plants were hypersensitive to high-salt and osmotic-stress conditions (Pandey et al., 2015). This manifested as inhibited root growth and a low rate of seed germination, both of which were more pronounced in the mutant plants than in the wild type. This hypersensitivity was attributed to the loss of function of the *cipk21* gene in the mutant plants (Pandey et al., 2015). This suggests that CIPK21 mediates a plant's response to salt-stress conditions by regulating ion and water homeostasis across the vacuolar membranes (Pandey et al., 2015). In an earlier study, the overexpression of *ZmCIPK21*, in Arabidopsis under salt stress conditions, reduced Na^+ accumulation and enhanced K^+ retention, thereby enhancing plant salt tolerance (Chen et al., 2014). Another study showed that *MdCIPK24*/*MdSOS2* in apple plants was up-regulated by $CaCl_2$ and interacted with CBL1, 4, and 10 (Hu et al., 2016), When *MdCIPK24* was induced in tomato plants to evaluate its function in salt tolerance, the results revealed that, under 300 mM NaCl, the transgenic tomato had a higher salt tolerance than the wild type, suggesting that the function of MdCIPK24 in salt tolerance is conserved in different species. In addition, an increase in antioxidant metabolites, such as procyanidin and malate was also observed in *MdCIPK24*-overexpressing mutant apple plants (Hu et al., 2016). However, a few studies did reveal a negative regulation of plant salt tolerance by CBL-CIPKs. For example, *OsCIPK03*-overexpressing mutant plants exhibited hypersensitivity to salinity, during both seed germination and seedling growth, by their lower shoot K^+: Na^+ ratio, and a lower chlorophyll content, compared to the wild type. This indicates a possible negative effect of OsCIPK03 on salt tolerance in rice (Rao et al., 2011).

1.8.3.2 The role of CIPKs in osmotic stress signaling

The initial responses to water stress include rapid reduction in leaf growth rate, followed by partial or complete stomatal closure. These result in a reduction in the rate of transpiration and photosynthesis. Moreover, water stress inhibits root growth, and if the water stress is long-term, ultimately plant growth and yield are reduced (Neumann, 2011). In a bid to survive under such adverse environmental conditions, plants have evolved numerous responsive genes to restore cellular homeostasis. Various signaling proteins, such as transcription factors, protein kinases and phosphatases, play important signal-transduction roles under abiotic stress conditions, by activating adaptive downstream responses that promote plant growth (Golldack et al., 2014).

In the rice genome, 15 genes of the CIPKs family were induced by drought stress, in a study by Xiang et al. (2007). Of these, the *OsCIPK12* gene was the one shown to play a positive role in rice drought tolerance. This was because the*OsCIPK12* overexpressing mutant plants' ability to accumulate higher proline and soluble sugar content, thus exhibiting better survival rates under drought stress, compared to the wild type (Xiang et al., 2007). Another gene, *OsCIPK23,* was also shown to mediate rice drought tolerance (Yang et al., 2008). In this study, the suppression of *OsCIPK23* expression significantly reduced seed set and increased the rice plants' hypersensitivity to drought stress. It was also noted in the same study that the expression of several drought-stress related genes was consistently induced when the *OsCIPK23* gene overexpressed in rice plants.

In Arabidopsis, *CIPK9* expression was up-regulated rapidly after 1 h, reaching a maximum both at 6 and 12h of exposure to osmotic stress (mannitol), and then once again falling after the 24th hour. However, phenotypic analysis, both of the seedlings and adult plants under drought stress, did not reveal any significant differences between the *CIPK9* mutant plants and wild type (Pandey et al., 2007). CBL1 and CBL9 interact with CIPK23, in plasma membrane, *in vivo,* and their function was detected in various tissues including guard cells and root hairs (Cheong et al.,

2007). In a study using reverse genetic screening, loss of function of *cipk23* was found to reduce transpiration, by regulating the stomatal movement in an ABA-dependent manner. Moreover, *cipk23* mutant plants suffered a significant degree of growth impairment, accompanied by a reduction in the efficiency of K^+ uptake by the roots, when they were grown in K^+-deficient conditions (Cheong et al., 2007). Similar morphological and physiological responses have been observed in *akt1* mutant plants subjected to long-term water stress (Nieves-Cordones et al., 2012). Both of these studies suggest that the CBL1 and the 9-CIPK23 complex negatively regulated AKT1, the K^+ transporter that is in root and stomatal guard cells, resulting in increased plant sensitivity to water stress (Cheong et al., 2007; Nieves-Cordones et al., 2012). Another study characterised the expression of *CIPKs* genes in maize under water stress, showing that five *ZmCIPK1*, *3*, *8*, *17*, and *18* genes were regulated by PEG, $CaCl_2$, ABA, and H_2O_2, and that their expression was affected by ABA and H_2O_2, in an organ-dependent manner (Tai et al., 2013).

1.8.3.3 The role of CIPKs in plant adaptation to K^+-deficiency

As an essential macronutrient for plant growth, and development, potassium has two main functions in plant cells: a biophysical one, such as in osmoregulation, and a biochemical one, such as in protein synthesis and enzyme activation (Hakerlerler et al., 1997; Leigh and Wyn Jones, 1984). The concentration of K^+ in soil solution varies widely within a range of 0.1-6 mM. Larger quantities of K^+, however, can be accumulated by the plant, and may constitute between 2 and 10% of plant dry weight (Leigh and Wyn Jones, 1984). Potassium-deficiency symptoms appear when potassium constitutes less than 10% of plant dry weight. Cytoplasmic K^+ concentration is, however, maintained at approximately 100 mM (Leigh and Wyn Jones, 1984), while the vacuole retains a high K^+ concentration range, of between 20-500 mM, for use under low K^+ conditions (Walker et al., 1996). This variability in the vacuolar K^+ concentration reflects the potassium status of the plant. Under sufficient supply, the vacuole retains up to 200 mM for the maintenance of cytoplasmic K^+ concentration. Under K^+-deficiency conditions, however, it has been reported that

vacuolar K^+ drops to 10-20 mM; a concentration at which it is no longer able to maintain cytosolic K^+ (Leigh and Wyn Jones, 1984; Walker et al., 1998). A significant number of selective and non-selective channels and transporters, localised in the plasma membrane and tonoplast, confer the K^+ uptake efflux and long-distance transport (Shabala and Pottosin, 2014). Potassium-uptake functions can be performed either by a high-affinity mechanism, which is conferred by their transport function at low external K^+ concentration, of less than 0.2 mM (Epstein et al., 1963; Maathuis and Sanders, 1994), or by a low-affinity mechanism which is conferred by K^+ channels at a high external K^+ concentration, of above 0.2 mM (Epstein et al., 1963).

It has been reported that the activities of ion channels and transporters are regulated to a major extent by phosphorylation and de-phosphorylation (Lee et al., 2007; Ragel et al., 2015). Low-affinity, inwardly-rectifying K^+ channels, such as the Arabidopsis K^+ transporter AKT1, has been shown to be regulated by CBL-CIPKs (Lee et al., 2007; Li et al., 2006). Of these, *CIPK23*, *6*, and *16* phosphorylated and activated AKT1 in a BCL1-dependent manner, with *CIPK23* being the more effective gene. In contrast, the 2C-type protein phosphatase (PP2C), interacting with CIPK23, dephosphorylated and inactivated the AKT1 channel in the plant root (Lan et al., 2011; Lee et al., 2007). A model of the signaling pathway by which plants respond to low K^+ conditions, has been proposed. According to this model, under low K^+ conditions, calcium signature activates CBL and CBL2 calcium sensors that interact and activate the protein kinase, *CIPK23*. The CBL-CIPK23 complex phosphorylates and activates AKT1, thereby enhancing K^+ uptake in plants (Li et al., 2006). It has been consistently shown that loss of function of *cipk23*, *cbl1* or *cbl9* leads to increased plant hypersensitivity to low K^+ conditions (Xu et al., 2006). Since *OsAKT1* is modulated by the OsCBL1-OsCIPK23 complex, loss of function of either *Oscipk23* or *Osakt1* under a low K^+ condition causes the appearance of similar K^+-deficiency symptoms (Li et al., 2014). Under a low K^+ condition, CIPK23 is purported to activate the high-affinity K^+ transporter, HAK5. Under this condition (10μM), CBL1, 8, 9, 10 Ca^{2+} sensors activate CIPK23, which

phosphorylates the N terminus of *HAK5*, up-regulating its activity; this results in an increase in K^+ influx (Ragel et al., 2015). Another study demonstrated that the CBL4-CIPK6 interaction modulates the activity of the AKT2 K^+ channel in plant cells, by translocating the AKT2 from the endoplasmic reticulum membrane to the plasma membrane and enhancing AKT2 activity in oocytes (Held et al., 2011). As this translocation is dependent upon dual lipid modifications of CBL4 (by myristoylation, and palmitoylation, respectively), the Ca^{2+} sensor modulates K^+ channel activity in a kinase interaction-dependent and/or phosphorylation-independent manner (Held et al., 2011). The results of transcriptome analysis of rice root response to potassium deficiency showed that eight *OsCIPK* genes (2, 6, 9, 10, 14, 15, 23, 26) were up-regulated, while two others, *OsCIPK29* and *OsCIPK31,* were down-regulated (Ma et al., 2012b). The transcript of *CIPK9,* a gene that is responsive to abiotic stress conditions, was induced in both the root and shoot of Arabidopsis, under a low K^+ condition (Pandey et al., 2007). It was also detected under a normal K^+ condition (MS medium) (Liu et al., 2013). Although both K^+ uptake and K^+ content in the mutant plant remained unaffected by a low K^+ condition ($\leq$ 20µM), the disruption in the function of the *Oscipk9* increased K^+ plant hypersensitivity to the K^+-deficient condition (Pandey et al., 2007). In addition, *cipk9* mutant plants did not display any phenotypic change compared to the wild type, both under salt- and osmotic-stress, suggesting that CIPK9 has a function that is related specifically to K^+. The double mutation in the tonoplast-localized CBL proteins, *cbl2* and *cbl3,* resulted in stunted plant growth with leaf-tip necrosis, reduced root development and seed set, and reduced seed weight and fatty acid content (Eckert et al., 2014; Tang et al., 2012a); these are typical symptoms of nutrient deficiencies under normal growth conditions. Moreover, the double mutant *cbl2cbl3* showed hypersensitivity to an excessive level of external ions, and reacted by altering the plants' ionic profile to one that was more specific to K^+ accumulation. At the same time, this caused adefect to occur in the activity of V-ATPase, which has been shown to play a role in Na^+ sequestration in the vacuoles of plant cells (Tang et al., 2012a). In contrast, another

study showed that the overexpression of *CIPK9*, *CBL2*, and *CBL3* in Arabidopsis plants under a low K^+ condition, decreased their chlorophyll and K^+ content, compared to the wild type, resulting in a low-K^+ sensitive phenotype (Liu et al., 2013). When grown under a low K^+ condition, both the *cbl3* and *cipk9* mutants consistently showed a similar low K^+-tolerant phenotype, which still had green shoots and a higher chlorophyll and K^+ content, compared to the wild type (Liu et al., 2013). Moreover, this study established that both *CBL3* and *CBL2* interact with *CIPK9* to up-regulate its expression in the tonoplast. Further analysis revealed strong expressions of *CBL2*, *CBL3*, and *CIPK9* in root vascular bundles. The root/ shoot K^+ ratio was also much more lower in the *cbl3*, and *cipk9* mutants, compared to wild type, under a low K^+ condition, suggesting that these genes have a role in K^+ translocation (Liu et al., 2013). However, the regulatory mechanism of the CBL3/CIPK9 complex in response to abiotic stress is not well understood, because neither the targeted proteins of the K^+ channel nor the K^+ transporters have been identified. In addition, when the previous studies tested the interaction between CIPK9 and K^+ transporters, and channels, such as AKT1, AKT2, HAK5, SKOR, TPK1, the results were negative (Liu et al., 2013; Pandey et al., 2007). Therefore, further studies are clearly needed to identify the role of CIPK9 in K^+ homeostasis.

The potential roles of CBL-interacting protein kinases (CIPKs) in ion homeostasis in response to abiotic stresses such salinity, drought, K^+-deficiency were summarized in (Fig. 1.4).

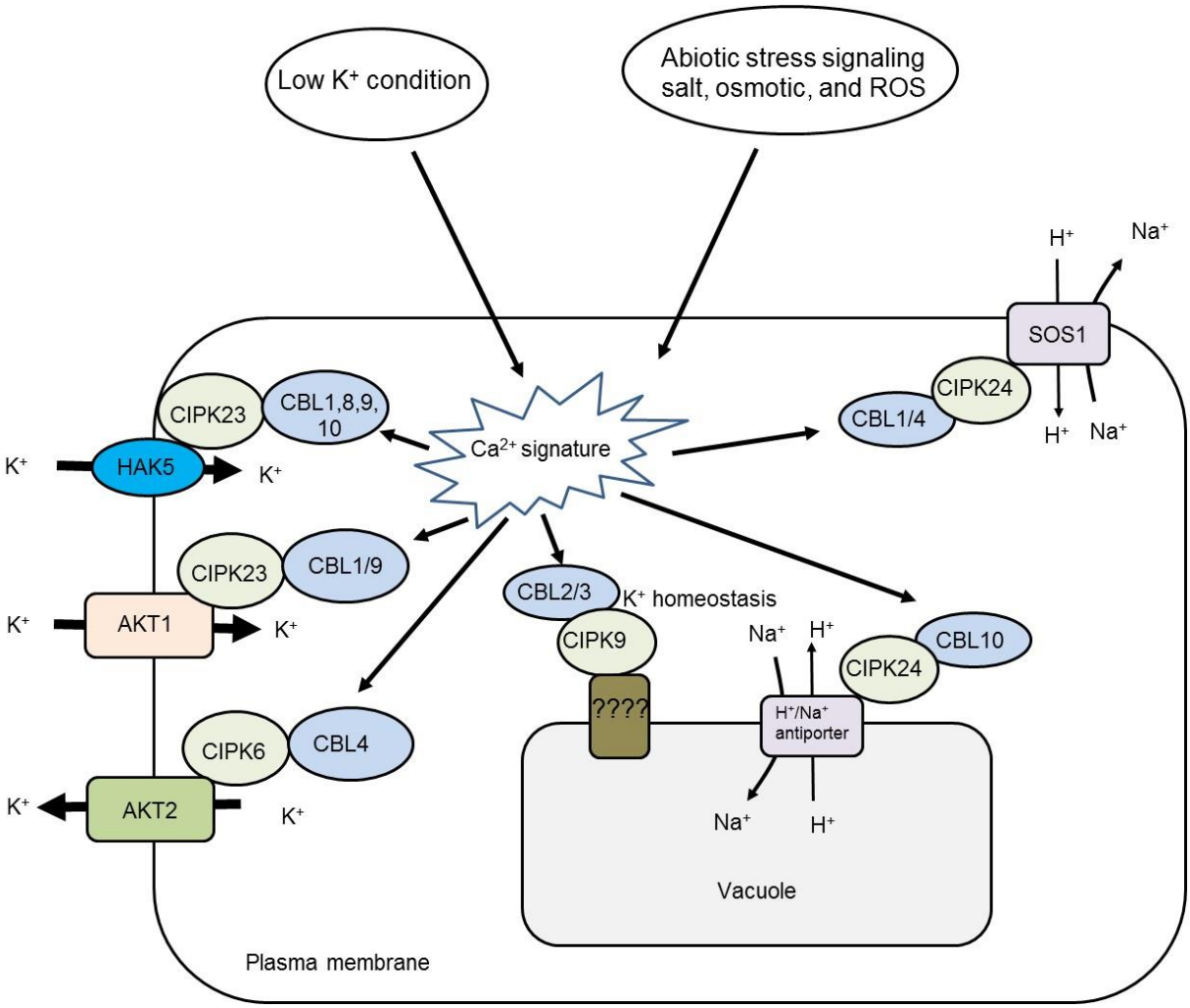

Figure 1. 4 Abiotic stress due tosalt, drought, ROS, and low K^+ conditions, induce calcium signals that activate calcineurin B-like proteins (CBLs). Different CBLs activate the same, or different CBL-interacting protein kinases (CIPKs), which, in turn, phosphorylate and activate different types of ion channels and transporters localised in the plasma membrane and tonoplast of plant cells, to maintain ion homeostasis. HAK5 (high-affinity potassium transporter 5), AKT (Arabidopsis K^+ transporter 1), AKT2 (Arabidopsis K^+ transporter 2), SOS1 (salt overly sensitive 1, a Na^+/H^+ antiporter (adapted from (Zhu et al., 2013).

The aims of this study

K^+ is a major inorganic macronutrient that is required by plants for growth and development. Because K^+ is involved in many of metabolic processes such as enzyme activity, protein synthesis, photosynthesis, membrane potential, pH, and ion homeostasis (Marschner and Rengel, 2012), plants take up large quantities of potassium. However, this is difficult for plants to do as the K^+ concentration in the soil solution is known to be low within an measured range of 10^{-5} to 10^{-3} M (Marschner and Rengel, 2012).

While Na^+ is a non-essential element for most plants, it may play a beneficial role in osmotic adjustment, particularly under a K^+-deficient condition. However, it has been clearly demonstrated that excessive levels of Na^+ in the growth medium negatively impact plant growth and limiting K^+ uptake by the roots. Salt tolerance in plants is linked to their ability to maintain a high K^+: Na^+ ratio by simultaneously restricting Na^+ uptake and transport within the plant and improving K^+ uptake. This study addressed the role of NH_4^+ in the availability of ions and, consequently, the plant's ability to uptake Na^+ and K^+. In summary, the general aim of this study is to identify the electrophysiological roles of various K^+ and Na^+ transport genes in a glycophytic crop (rice) in order to deepen the current knowledge of the functions of the various components of the salt tolerance mechanism in such plants with the long-term goal of enabling the development of additional salt and drought tolerance traits.

The specific aims of this study were as follows:

1- To identify the role of OsCIPK9 in maintaining K^+ homeostasis in rice plants. The questions in this chapter were (1) how does the loss of function of *Oscipk9* influence the growth, ion concentrations, and the development of rice plants, under salt and drought stresses, and in the presence of various Ca^{2+} and K^+ concentrations?; and (2) how does the loss of function of *Oscipk9* alter the K^+ flux, in response to oxidative stress?.

2- To understand the role of the high-affinity OsHAK1 and OsHAK5 transporters in K^+ homeostasis in response to salt stress. To achieve this aim, the knock out *Oshak1* and *Oshak5* mutants were used to reveal the role of OsHAK1 and OsHAK5 in K^+ homeostasis in response to salt and oxidative stresses both, under high-affinity and low-affinity K^+ concentrations.

3- To investigate the role of OsAKT1 in Na^+ and NH_4^+ uptake under K^+-deficient conditions, as well as to gain a better understanding of the role of NH_4^+ in inhibiting the ability of OsAKT1 to uptake Na^+. The study used the knocked-out and the overexpressed *AKT1* mutants to address the functional role of AKT1 in ion-uptake, under a K^+-deficient condition.

4- To understand the role of *OsHKT1;5* gene in the long-distance transport of Na^+ and more broadly in the K^+ and Na^+ homeostasis in the rice plants. The main aim of this study was to investigate whether knocked-down of *OsHKT1;5* improves rice salt tolerance and its role in altering the K^+ and Na^+ flux and content in the rice plants.

Chapter 2

The signaling role of the calcineurin B-like protein-interacting protein kinase 9 (CIPK9) in K^+ homeostasis under K^+-deficient condition

2.1 Introduction

An understanding of plant responses to abiotic stress is vital for the genetic engineering of crop plants. This involves understanding the mechanisms by which plants perceive the stress-induced signal and generate the second messengers, such as Ca^{2+} and ROS (Lourenço et al., 2016; Mori and Schroeder, 2004; Steinhorst and Kudla, 2013), which can modify the intracellular Ca^{2+} concentration, producing what is known as Ca^{2+} signature (Knight et al., 1996; Sanders et al., 2002). In turn, this causes a protein phosphorylation cascade that finally targets proteins directly involved in cellular protection, or transcriptional factors regulating stress-induced genes (Xiong et al., 2002). Salinity (Kader and Lindberg, 2010; Laohavisit et al., 2013; Reid et al., 1993), drought (Hong-Bo et al., 2008; Knight et al., 1998; Pandey et al., 2007; Zhu, 2002b), and K^+ deficiency (Pandey et al., 2007) have been shown to induce transient Ca^{2+} influx, thereby increasing cytosolic Ca^{2+} concentration. This change in cytosolic Ca^{2+} levels can be detected by a number of high-affinity calcium sensors. In higher plants, several families of Ca^{2+} sensors have been recognised, including calmodulin (CaM) and CaM-related proteins (Snedden and Fromm, 2001; Zielinski, 1998), Ca^{2+} dependent protein kinases (CDPKs) (Harmon et al., 2000; Roberts and Harmon, 1992) and calcineurin B-like (CBL) proteins and their interacting protein kinases (CIPKs) (Kudla et al., 1999). CBL-CIPKs interacting transduces downstream signals that activate plasma membrane ion channels, pumps, and transporters, which in turn, facilitate the ion homeostasis by controlling ion uptake, long-distance transport, and sequestration (Thoday-Kennedy et al., 2015). Bioinformatics data have revealed a complement of 10 CBLs and 26, and 30CIPKs in Arabidopsis, and rice genomes, respectively (Kolukisaoglu et al., 2004; Weinl and Kudla, 2009). A characterisation

study of stress-responsive *OsCIPK* genes in rice showed that 15, 12, 12, and 16 of the *OsCIPKs* genes were induced by drought, salinity, PEG, and ABA treatments, respectively (Xiang et al., 2007). Moreover, the CBLs-CIPKs complex is not only involved in abiotic stress, but may also play a vital role in the developmental processes of rice plants (Kanwar et al., 2014). The salt overly sensitive (SOS) pathway is an example of a CBL-CIPK signaling pathway in response to salt stress. SOS3/CBL4 has been identified as a Ca^{2+} binding protein (Kudla et al., 1999). In response to salt stress, the transient alleviation in the cytosolic Ca^{2+} concentration activates SOS3/CBL4, which then interacts with SOS2/CIPK24 to directly regulate the downstream component of SOS1, a putative Na^+/H^+ antiporter, with the final result being the maintenance of low intracellular Na^+ (Qiu et al., 2002). Moreover, the SOS3-SOS2 complex may regulate the tonoplast Na^+/H^+ antiporter, to compartmentalise further Na^+ in vacuole, thus lowering Na^+ concentration in the cytosol (Qiu et al., 2004). In addition, SOS2/CIPK24 may modulate the plasma membrane H^+/Ca^{2+} antiporter (CAX1) to control intracellular Ca^{2+} homeostasis (Cheng et al., 2004). The first molecular and biochemical characterisation of the Na^+ efflux protein in monocot rice showed that the Arabidopsis protein kinase complex SOS2/SOS3 phosphorylated OsSOS1, thereby reducing the net Na^+ cellular content. Moreover, OsCIPK24 and OsCBL4 were able to activate OsSOS1 in yeast cells (Martínez-Atienza et al., 2007). However, another study indicated that OsCIPK03 negatively regulates salt tolerance in rice plants (Rao et al., 2011). In a study on the role of overexpression of the wheat *TaCIPK29* gene in tobacco salt tolerance, the results indicated that overexpression increased salt tolerance, since it maintained both a high K^+:Na^+ ratio and Ca^{2+} content, elevated the expression of *NtSOS1*, *NtNHX2*, *NtNHX4* and *NtCAX3*, improved antioxidant enzyme activities and reduced H_2O_2 content (Deng et al., 2013).

The CBL1/CBL9-CIPK23 complex regulates the AKT1 pathways that play a key role in K^+ homeostasis under water stress. A loss of function of *Atcipk23* and *cbl1/cbl9* resulted in plant tolerance to drought. The increase of this tolerance was due to the hypersensitivity of stomata to

ABA, which in turn caused a reduction in transpiration rate, while application of ABA enhanced stomatal closure in the *akt1* and *cipk23-5* mutants compared to the WT. Moreover, K^+ content was lower in the mutant plants in a growth medium with a K^+ concentration of 1.4 mM. This study suggested that AKT1 plays a substantial role in stomatal movement in response to water stress (Nieves-Cordones et al., 2012).

Another study showed that *AtCIPK6* was induced by salt and drought stresses, while its overexpression in Arabidopsis enhanced salt tolerance, it also increased sensitivity to ABA (Chen et al., 2013). In rice plants, OsCBL1-OsCIPK23 upstream regulates OsAKT1, which is responsible for K^+ uptake in rice roots. It has been reported that loss of function of *Oscipk23* caused similar K^+ deficient symptoms as occurred in the *Osakt1* mutant under low K^+ conditions. This suggested the critical role of AKT1 modulated by OsCIPK23 in K^+ homeostasis maintenance in rice plants (Li et al., 2014).

A recent study proved that CBL1, 8, 9, 10-CIPK23 interacting activates AtHAK5 *in vivo*, resulting in high-affinity K^+ transport in Arabidopsis roots (Ragel et al., 2015) Characterisation of the expression of five *CIPKs* genes in maize is a good example of the role of H_2O_2 and ABA signaling, under water stress. The results indicated that the expressions of *CIPK1*, *3*, *8*, *17* and *18* have been up-regulated by PEG, $CaCl_2$, ABA, and H_2O_2, respectively (Tai et al., 2013). In addition, H_2O_2 mediated the ABA-induced increase in *ZmCIPK1* expression in the leaves and roots, while H_2O_2 mediated the PEG-induced increase in *ZmCIPK1* expression, but in the roots only. This suggests that a H_2O_2-independent signaling pathway plays a role in water stress induced gene regulation, as well as in the H_2O_2-dependent signaling pathway.

The functional role of CIPK9 (Locus At1G01140), a member of CIPKs family, was investigated in transgenic Arabidopsis, both under abiotic stresses and low K^+ conditions. *AtCIPK9* expression was ubiquitous with a strong expression detected in the mature zone of the root, and, to a lesser extent, in its elongation zone (Pandey et al., 2007). It was also detectable in

the anther, stigma, petals and sepals, and in the majority of tissues of the young plants. Moreover, its expression was inducible under abiotic stress as well as under low K^+ conditions (Pandey et al., 2007). The strongest induction was by osmotic stress followed by high salinity, cold, then wounding (Pandey et al., 2007). The *Atcipk9* mutant plants were hypersensitive to K^+-deficient conditions, the growth both of their roots and shoots being significantly inhibited compared to the WT. Interestingly, no significant difference in K^+ uptake and content was observed in the *Atcipk9* mutant plants cultivated both in high K^+ (20 mM), and low K^+ (0.02 mM) growth media (Pandey et al., 2007). Another study that investigated the role of CIPK9 in K^+ homeostasis, under low K^+ conditions, concluded that CIPK9 interacts with CBL2 and CBL3, mainly in the tonoplast of Arabidopsis, resulting in K^+ homeostasis (Liu et al., 2013).

A recent study supported this adding that AtCBL2 and AtCBL3 interact with AtCIPK3, 9, 23 and 26 in the tonoplast, thereby regulating the transport proteins responsible for the vacuolar partitioning of Mg^{2+} (Tang et al., 2015). The results showed that *cbl2* and *cbl3* mutant plants were hypersensitive to external Mg^{2+}, in a dosage-dependent manner, specifically in the presence of a low Ca^{2+} concentration in the growth medium. This hypersensitivity was due the reduction in the ability of vacuole to sequestrate Mg^{2+} (Tang et al., 2015). However, exactly which proteins are targeted by CIPK9 is not fully known.

Several studies revealed that the AtCIPK9 has shown to affect K^+ transport in Arabidopsis (Liu et al., 2013; Pandey et al., 2007). So, this study aims to validate this fact on rice plants, with taking in consideration in the most cases CIPKs are labeled based on the number in the genome. It is often that CIPKs with the same number in different species can be different in their function, even they share reasonable level of sequence similarity and identity. Up to date, there is no a comprehensive study reported the function of *OsCIPK9* gene in rice plants in response to abiotic stress. On the light of these facts, this study will test if OsCIPK9 is involved in the regulation of K^+ transport in rice plants and aimed to fill the above gaps in our knowledge and reveal the role

of OsCIPK9 as a component of the abiotic signaling pathways and its impact on growth, ionic relations, and whole-plant physiological characteristics of rice plants grown, under a range of abiotic stress conditions, such as salinity, drought, and oxidative stress.

2.2 Materials and Methods

2.2.1 Plant material and growth conditions

Mature seeds of rice plants, *Oryza sativa L. Japonica* cv Dongjin wild type, and its mutant *Oscipk9* were obtained from Rice Functional Genomics, National Institute of Agricultural Biotechnology, Korea.

Seeds were surface-sterilised with 1% v/v sodium hypochlorite (commercial bleach) for 10 min, and then thoroughly rinsed with sterile deionised water at least five times. Seeds were sown in sand, and then incubated at 28 °C and 100% relative humidity, and kept in darkness for 5 days, until germination. The seedlings were then transferred to a 5-litre hydroponic system, consisting of a number of containers made of black plastic, so as not to reflect light, each holding 9 plants. Hoagland solution was used as the growth medium [1.25 mM KNO_3; 0.5 mM $Ca(NO_3)_2$, 0.5 mM $MgSO_4$; 42.5 μM Fe-EDTA; 0.625 mM KH_2PO_4; 0.16 μM $CuSO_4$; 0.38 μM $ZnSO_4$; 1.8 μM $MnSO_4$; 45 μM H_3BO_3; 0.015 μM $(NH_4)_2MO_7O_{24}$; 0.01 μM $CoCl_2$ (pH 5.5–6.0)]. Containers were placed in the growth room that was set on a light/dark cycle of 16/8h daily with irradiance of 300 μmol m^{-2} s^{-1} at a day/night temperature of 28/20 °C, and with a relative humidity of 60-75% for a further one week. Containers were then transferred to the glasshouse where conditions were controlled with the temperature maintained at 28/ 20 °C day/night, and a high relative humidity ≥80%. Two mercury vapour (2X400w) lamps were set to provide 16-hour days. The experiment design was randomised complete block design with three replicates, each container (replicate) held nine plants for each treatment. The nutrient solution was changed every seven days to ensure ion compensation and root aeration. The rice seedlings were exposed to different abiotic stress conditions for 3 weeks, as described below.

2.2.2 Treatments

Three experiments were conducted to study the effect of loss of function of *Oscipk9* on plant's growth and development, under varius environmental conditions. Firstly, eleven-day-old seedlings of wild type (Dongjin), and its mutants *Oscipk9* were exposed to two levels of salinity (moderate 40 mM, and severe stress 80 mM of NaCl) for three weeks. A preliminary experiment was conducted, the aim of which was to determine the strength and duration of the salinity condition, under which the leaves of the plants remained green, so as to be able to determine the morphological and physiological characterization at the end of the experiment.

In the second experiment, osmotic stress was implemented by addition of 11.8% (w/v) of polyethylene glycol 4000 (PEG4000) (isotonic to 80 mM NaCl), imposing an osmotic stress of 0.362 MPa (Mexal et al., 1975; Yamane et al., 2003).

In the third experiment, seedlings of the wild type, and its mutant were exposed to two levels of potassium. The first level was K^+-deficiency (0.1 mM KCl), while the second was a high K^+ concentration (10 mM KCl). Two different amounts of calcium were also added (0.1 and 1.5 mM $CaCl_2$). All treatments were continued for 3 weeks. To create the low K^+ treatment, all potassium salts in the control medium were replaced with equimolar quantities of the corresponding sodium salts.

2.2.3 Whole-plant physiological assessment

After 3 weeks of treatments, measurements of chlorophyll content and stomatal conductance were made at different locations on 6 randomly-selected matured and fully-expanded leaves of each treatment. These measurements were made on days of full sun at between 11:00 am and 1:00 pm, in order to minimise diurnal influences, and within a temperature range of 23-30 °C. The chlorophyll content was measured using a Minolta Chlorophyll Meter SPAD-502 (Konica Minolta, Osaka, Japan), and stomatal conductance, by means of a Decagon leaf porometer

(Decagon Devices Inc., WA. Australia). At the end of the experiment, the numbers of dead leaves and tillers were also counted per plant. For the destructive measurements, plants of all lines were harvested and their fresh weight was immediately noted. The plants were then dried at 65 °C, in a Unitherm dryer for 3 days, in order to ensure a consistent weight (Birmingham, UK), then weighed to determine their dry weight.

2.2.4 Osmolality and ion content

Leaf and root osmolality, and K^+ and Na^+ content were determined using the freeze-thaw method described by (Tomos et al., 1984). Briefly, this method involved the roots and 3 leaves of each line/treatment being immediately harvested, then rinsed in 10 mM $CaCl_2$ to remove the apoplastic Na^+ (Shabala et al., 2016), then blotted on tissue paper. Next, the samples were placed into 1.5 mL microfuge tubes and stored at -20°C for at least 24 h. The samples were subsequently thawed, and the sap squeezed from the tissues using a pointed glass rod. A small portion (10µL) of these sap samples was used for osmolality determination using a vapour pressure osmometer (Vapo, Wescor Inc. Logan, Utah, USA). The remainder of the sap samples were diluted x100 times with distilled water , and the K^+ and Na^+ contents of the leaves and roots were measured using a flame photometer (Model PFP7 flame photometer, Jenway, Bibby Scientific Ltd, UK).

2.2.5 Non-invasive ion flux measurements using MIFE system

2.2.5.1 Theory of the MIFE technique

The theory of the MIFE system, as described by (Newman, 2001) is based on the principle that ions tend to move by diffusion in solution from a high to low concentration, and, via electrical forces, from a high electrochemical potential to a low. Thus, if the combined electrochemical potential gradient can be measured, the net ion flux by diffusion can be calculated, by applying the Nernst equation to this gradient as well as the mobility and concentration of the ions in the solution.

$$j = cuzfg\left(\frac{dv}{dx}\right)$$

Where j = ion flux (nmol m^{-2} s^{-1}); c = ion concentration (mol m^{-3}); u = ion mobility (m s^{-1} per Newton mol^{-1}); z = ion valence; f = Faraday number (96500 C mol^{-1}); g = factor found from the measured Nernst slope for the electrode during calibration; dv = voltage difference measured by an electrometer between the two positions (V); dx = distance between the two positions (Fig. 2.1).

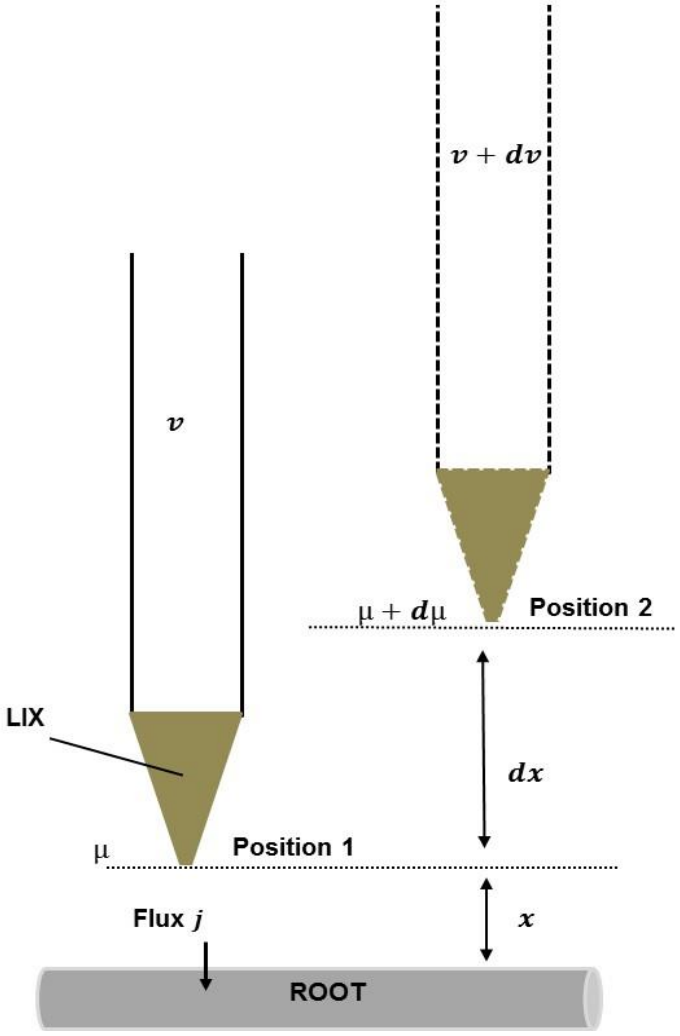

Figure 2. 1 Illustration of MIFE theory. The microelectrode tip is filled with liquid ion exchanger (LIX), and moved between two positions (dx). Position one is near the epidermal cells of rice roots, and position two is 50 µM distance away from position one. The electrode voltage, (dv) between the two positions is measured with an electrometer; μ is the electrochemical potential of proposed ion in the solution (joules mol^{-1}); j is the net ion flux (nmol m^{-2} s^{-1}) (Newman, 2001).

Theoretically, if the microelectrode, with a specific ion exchange liquid (LIX) in its tip, were at position one, which is close to the plant tissue, and the concentration of the targeted ion at position 1 were lower than at position 2, which is further away, this means that the net movement of ions would be influx. Conversely, if the concentration of the targeted ion at position 1 were to increase more than that of the targeted ion at the more distant position 2, then the net movement of ion is efflux (Newman, 2001). The ion flux is associated with the specific location of tissue surface, as well as the geometry of the tissue at that point. In the case of this study, the ion flux measurements were done on the epidermal cells of mature and elongation zones rice roots, which have a cylindrical geometry. Therefore, the root diameter, and the distance between the tissue surface and the microelectrode were both taken into consideration in calculating the flux (nmol m^{-2} s^{-1}) using MFLUX software (University of Tasmania, Hobart, Australia). The data were then transferred to an excel spreadsheet for further analysis.

2.2.5.2 Microelectrode fabrication

The microelectrode fabrication was carried out using a method developed by Shabala et al. (2006a) and Shabala et al. (2013) in the plant physiology laboratory at the University of Tasmania. The blank microelectrodes are made from non-filamentous, borosilicate glass capillaries (1.5-mm outer diameter) with an internal diameter of 0.86 mm GC 150-10, (Harvard Apparatus Ltd, Kent, UK). These blank microelectrodes were pulled to form tips with a diameter of < 3 μm using a vertical pipette puller (PP 830, Narishige, Tokyo, Japan), and were then placed upright, base down, on a stainless-steel rack, before being placed in an oven, where they were dried at 250°C, overnight. Ten to 15 min before salinisation, these electrodes were covered with a steel lid for 20 min, and then, 40–50 μL of salinizing agent, tributylchlorosilane (90796, Fluka Chemicals, Busch, Switzerland) were injected under the lid. After ten min, the lid was removed and the microelectrode blanks were then baked at 250°C for a further 30 min. By this stage, the surface of the blank microelectrodes blank became hydrophobic, thus, the hydrophobic liquid ion exchangers

(LIXs) could move to the tip of the prepared microelectrodes. Next, a LIX-containing tube, a glass microcapillary with a ~30–50-µm diameter at its tip was prepared by filling it with approximately 1 mm of the LIX stock cocktail (Table 2.1). The microelectrode blank was then mounted horizontally on a three-dimensional micro-manipulator, and then under a stereo microscope the electrode tips were flattened by gently pressing the blank against a flat glass surface to obtain a tip diameter of 2–3 µm. Once the correct tip size had been attained, the microelectrodes were filled with the appropriate back-filling solutions (Table 2.1) using a syringe with a thin, non-metallic needle. Next, the electrode tip was quickly front-filled with the corresponding LIX, (Table 2.1), by pressing it gently to the tip of the tube containing the LIX to achieve a column length between 100-150 µm from the tip. These prepared electrodes were subsequently left immersed in the measuring solution until they were used. It should be noted that prepared electrodes of potassium and calcium can be used immediately after preparation.

Table 2. 1 Ionophores (LIX) and the back-filling solutions which were used to prepare the microelectrode of the selected ion

Ion	Ionophore (LIX)	Back-filling solution (mM)
K^+	Valinomycin	500 KCl
Ca^{2+}	(-)-(R,R)-N,N′-(Bis(11-ethoxycarbonyl)undecyl)-N,N′-4,5-tetramethyl-3,6- dioxaoctanediamide	500 $CaCl_2$

2.2.5.3 Reference electrode

A silver wire was chlorided (Ag/AgCl) for 15 min using 0.25 N HCl, and was then inserted into a broken glass micro-capillary, with a 50 µm tip diameter, filled with 1 M KCl in 2% agar. The micro-capillary was then held together by being tightly bound with parafilm strips (Shabala et al., 2013).

2.2.5.4 Calibration

An appropriate set of three standard solutions, (Table 2.2) covering the expected range of targeted ion was prepared. A calibration of the microelectrodes was then performed, using the MIFE CHART calibration. In doing this, it was taken into consideration that the most efficient electrodes must have a slope above 50 mV per decade for monovalent ions, and one of at least 25 mV per decade for divalent ions, and that the correlation coefficients for the three points of standard solutions must be ≥ 0.999 (Shabala et al., 2013).

Table 2. 2 Measured ions and their standard calibration solutions

Ion	Standard calibration solutions
Ca^{2+}	100, 200, 400 µM $CaCl_2$
K^+	250, 500, 1000 µM KCl

2.2.5.5 Sample preparation for MIFE measurements

Rice seeds were germinated inside an incubator set at 28°C and 100% relative humidity, to insure a high success rate of germination. The growth media used for the *Oscipk9* experiment made up from four different combinations of potassium and calcium ions as follows: low K^+/high Ca^{2+}, low K^+/low Ca^{2+}, high K^+/high Ca^{2+}, and high K^+/low Ca^{2+} (low K^+ 0.5 mM, high K^+ 50 mM, low Ca^{2+} 0.1 mM, and high Ca^{2+} 1.5 mM). Roots of healthy 5-6-day-old seedlings were chosen and carefully placed on the centre of a glass holder and fixed firmly with Parafilm strips on both sides to avoid root movement during the measurement. The glass holder was then placed inside the measuring chamber that was partially filled with the bathing medium, BSM (Basal Salts Medium), consisting of 200 µM NaCl, 100 µM $CaCl_2$, and 200 µM KCl. The pH level of the BSM solution was maintained between 5.2 and 5.6. For conditioning, the roots were left in the bathing solution for approximately 30-60 min.

2.2.5.6 Experimental protocols

The measurement chamber was placed on a holder opposite the microscope. Then, the root came to the clear focusing by using a joystick manipulator, and aligned the tips of three microelectrodes at a distance of 50 µm from the epidermal cells of the rice roots, and with a 1-2 µm separation between each of the three. The data acquisition process was started, using the CHART program based on DOS. Data acquisition under the normal condition lasted for 5-7 min on the mature zone until steady-state fluxes were obtained. To study the response of the plants to oxidative stress the 5 mM of H_2O_2 treatment was quickly added without causing major disturbance to the plant root in the measurement chamber. Meanwhile, the distance between the electrode and the root surface was kept constant at 50 µm, and the distance between Position (1) and position (2) of the microelectrode was 50 µm for all the MIFE experiments. The data acquisition continued for a further 30 min. The ion fluxes were then calculated using MFLUX software, and the resulting data imported to an Excel spreadsheet for further analysis, and the data were presented in figures as a steady state fluxes (Shabala et al., 2013).

2.2.6 Statistical analysis

All data used in this thesis are expressed as mean values± SE. The statistical significance of mean and standard error values was determined by the t- test at $P \leq 0.05$ using SPSS software version 20 (IBM support portal, USA).

2.3 Results

To investigate the role of CIPK9 in the growth of rice plants, under various abiotic conditions, three separate experiments were conducted in a glasshouse to measure a range of morphological and physiological parameters.

The *Oscipk9* mutant showed a significantly ($P \leq 0.01$) higher dry weight by 47%, than the WT, under the control (non-saline condition) (Fig. 2.2a). In the *Oscipk9* mutant, the dry weight was not affected by exposure to moderate salinity (40 mM) for 3 weeks. However, under moderate salinity (40 mM), the dry weight of the WT significantly ($P \leq 0.05$) decreased by 24% compared to its counterpart (Fig. 2.2a). When the salt concentration was increased to 80 mM NaCl, a further reduction occurred in the dry weight of the WT and the *Oscipk9* mutant by 20 and 30%, respectively (Fig. 2.2a). Taken together, these results point to the absence of any significant difference in salt sensitivity between WT and the *cipk9* knockout under the high saline condition.

Under moderate salinity, the number of dead leaves increased by 3- and 11-fold, in the WT and the *Oscipk9* mutant, respectively (Fig. 2.2b). The significantly higher number of dead leaves in the *Oscipk9* mutant, compared to the WT, could be due to genetic transformation, which altered the genome sequence, causing a significantely higher fresh weight, number of tillers, and greater vegetative growth (data not shown) in the *Oscipk9* mutant, compared to the WT under the control condition. Severe salinity caused a 6-fold increase in the number of dead leaves in the WT, while in the *Oscipk9* mutant, this number increased by only 26% (Fig. 2.2b).

Under control condition, both lines had a similar stomatal conductance rate of approximately 70 mmol m^{-2} s^{-1} (Fig. 2.2c). However, stomatal conductance of both lines was significantly ($P \leq 0.01$) affected by salinity, rapidly decreasing when salinity was increased to 80 mM NaCl (Fig. 2.2c). There was also a pronounced and significant ($P \leq 0.01$) decrease in stomatal conductance in the WT and the *Oscipk9* mutant by 60 and 35% respectively. Under severe salt

stress, the *Oscipk9* mutant displayed a significantly ($P \leq 0.05$) higher stomatal conductance than the WT (Fig. 2.2c).

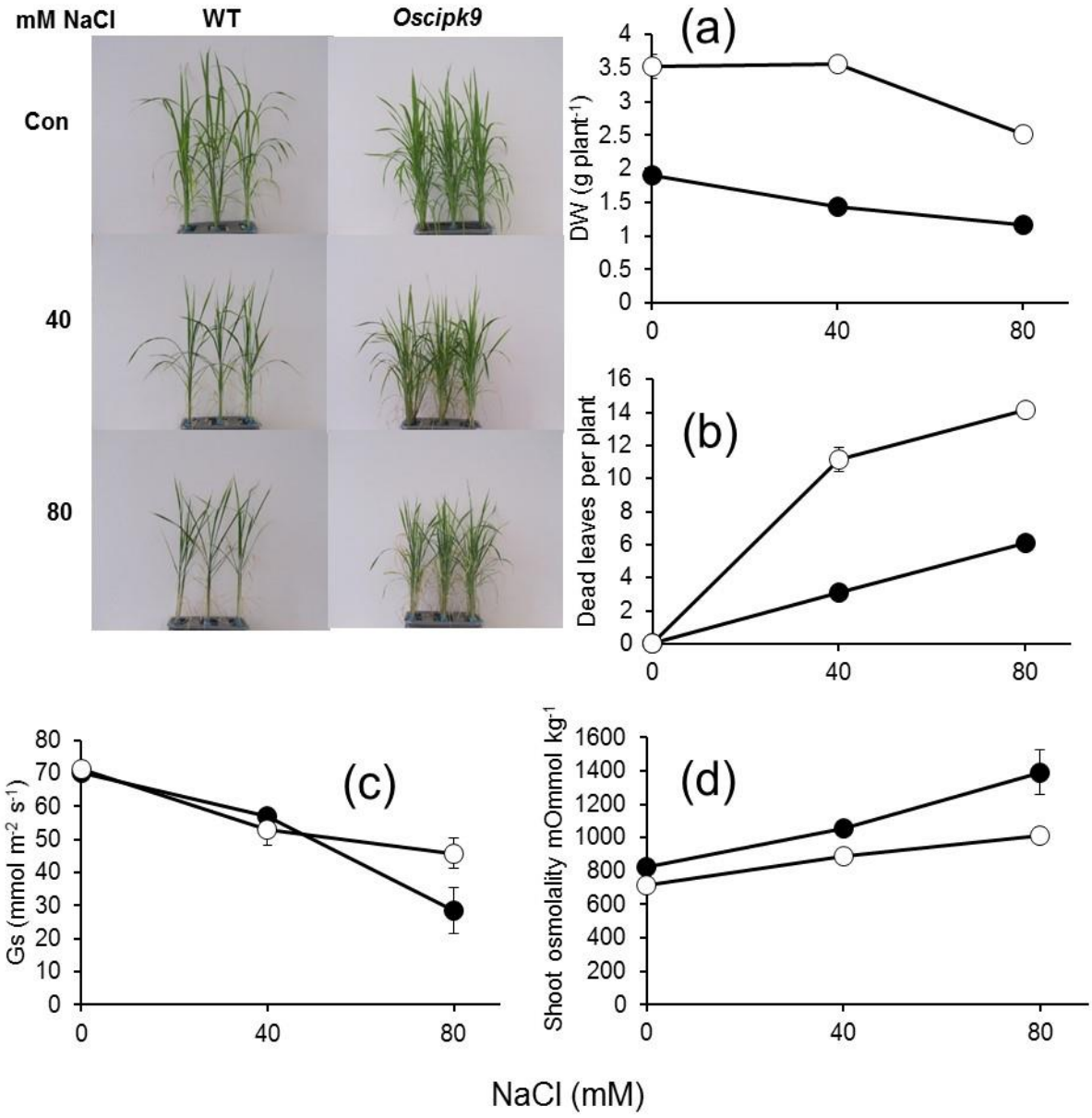

Figure 2. 2 Effects of two concentrations of NaCl (40, and 80 mM) on the growth of *Oscipk9* mutant plants and the WT compared to that of the control: (a) dry weight, (b) number of dead leaves per plant, (c) stomatal conductance, and (d) shoot osmolality. The plants of both lines were grown in a hydroponic system for 21 days. The WT (closed circles), *Oscipk9* mutant line (open circles). Data are the mean ± SE (n=18).

Both lines had the same shoot osmolality, under the control condition (Fig. 2.2d). However, the introduction of salinity significantly ($P \leq 0.01$) increased shoot osmolality in the WT and the

Oscipk9 mutant by 68 and 41%, respectively (Fig. 2.2d). It should also be noted that this osmolality increase was significantly (P≤0.01) lower in the *Oscipk9* mutant compared to the WT (Fig. 2.2d).

2.3.1 Ion content

The results of this study showed that while the *Oscipk9* mutant and the WT had a similar shoot Na^+ content (Fig. 2.3a), they both differed significantly in their shoot K^+ content when grown under the non-saline control condition (Fig. 2.3c), with the *Oscipk9* mutant showing a significantly ($P \leq 0.01$) lower (by 43%) shoot K^+ content compared to the WT. Exposure to salinity for 3 weeks altered both the Na^+ and K^+ content in both shoots and roots, in both lines. Under moderate salinity, the shoot Na^+ content gradually increased by 12-14-fold, but with no significant difference between the lines (Fig. 2.3a), while the shoot K^+ content was not affected and remained close to the level of those grown under the non-saline condition (Fig. 2.3c). However, when the plants were exposed to severe salt stress, their shoot Na^+ content increased dramatically by 71- and 88-fold in the *Oscipk9* and WT, respectively (Fig. 2.3a). Both lines showed a non-significant difference in their shoot Na^+ content, under severe salt stress. While their shoot K^+ content decreased by 25% in the *Oscipk9* mutant, it increased by 17% in the WT compared to the control condition (Fig. 2.3c). As a result, a significant shift in the K^+: Na^+ ratio under severe salt stress occurred, with this ratio decreasing by 75, and 94-fold in the WT, and the *Oscipk9* mutant, respectively compared to plants, under the control condition. Furthermore, this ratio was lower in the *Oscipk9* mutant than the WT (data are calculated based on the data presented in Fig. 2.3).

In terms of the Na^+ and K^+ content of their root, no significant difference was detected between the lines under the non-saline condition (Fig. 2.3b, d). However, under the moderately saline condition, the Na^+ content increased by 11-fold in both lines, accompanied by a rapid and significant decrease in the root K^+ content (Fig. 2.3 b, d). While increasing the salinity to a severe level caused no further increase in the root Na^+ content in the WT compared to the WT under the moderate salt stress condition, while a dramatic and significant ($P \leq 0.01$) increase (by 34-fold)

did occur in the *Oscipk9* mutant compared to the control condition (Fig. 2.3b). In addition, this mutant showed a non-significant decrease in its root K^+ content, while the WT showed an even further reduction in K^+ content under the severe salt stress condition (Fig. 2.3d).The root K^+: Na^+ ratio was significantly ($P \leq 0.01$) higher in the WT compared to the *Oscipk9* mutant under the control condition. This difference was further increased by salinity, with the increase being higher in the *Oscipk9* (60-fold) compared to WT line (26-fold).

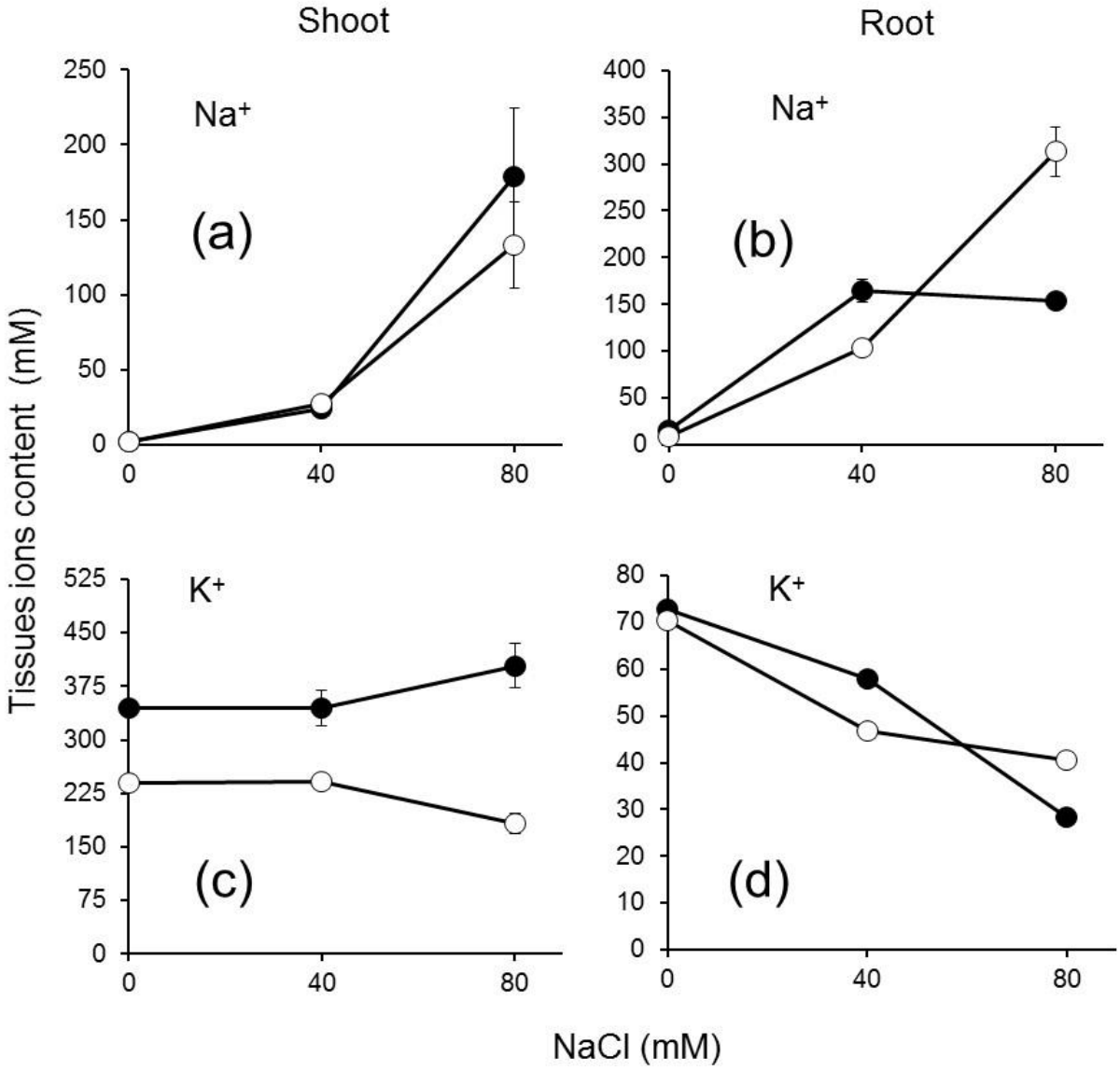

Figure 2. 3 The effect of salinity on (a) shoot Na^+ content, (b) root Na^+ content, (c) shoot K^+ content and (d) root K^+ content. Plants of both lines were grown under the control, 40, and 80 mM NaCl) in a hydroponic system for 21 days. The WT (closed circles), *Oscipk9* mutant line (open circles). Data are the mean ± SE (n=6).

2.3.2 Drought stress

Results of the drought stress experiment showed that the *Oscipk9* mutant experienced a stronger vegetative growth, and produced a larger number of tillers (data not shown). This resulted in a significant (by 40%; $P \leq 0.01$) increase in dry weight in the plants grown under the control condition (Fig. 2.4a). This increase was found to be unrelated to any of the plant's physiological features, as according to the SPAD value, the chlorophyll content was significantly ($P \leq 0.05$) lower in the *Oscipk9* mutant compared to the WT (Fig. 2.4c). Also, no significant difference in stomatal conductance (gs) was detected between the lines (Fig. 2.4d). While neither Na^+ content of the shoot and root nor root K^+ content differed significantly between the lines (Fig. 2.5a, b, d), shoot K^+ content was significantly lower in the *Oscipk9* mutant as compared to the WT under the control condition (Fig. 2.5c). As a result, both the shoot K^+: Na^+ ratio and shoot osmolality were significantly ($P \leq 0.01$) lower by 58%, and 27% in the *Oscipk9* mutant compared to the WT, respectively.

Drought stress caused a significant ($P \leq 0.01$) reduction of 39% in the dry weight of plants of both lines (Fig. 2.4a). A higher number of dead leaves was detected in the *Oscipk9* mutant compared to the WT, most likely as a result of the larger total number of leaves in this line (Fig. 2.4b). The chlorophyll content (SPAD) and stomatal conductance (g_s) of the *Oscipk9* mutant were not significantly affected by drought stress. However, both were significantly reduced by 16%, and 19%, respectively in the WT, implying a significant difference in response to drought stress between the lines (Fig. 2.4c, d).

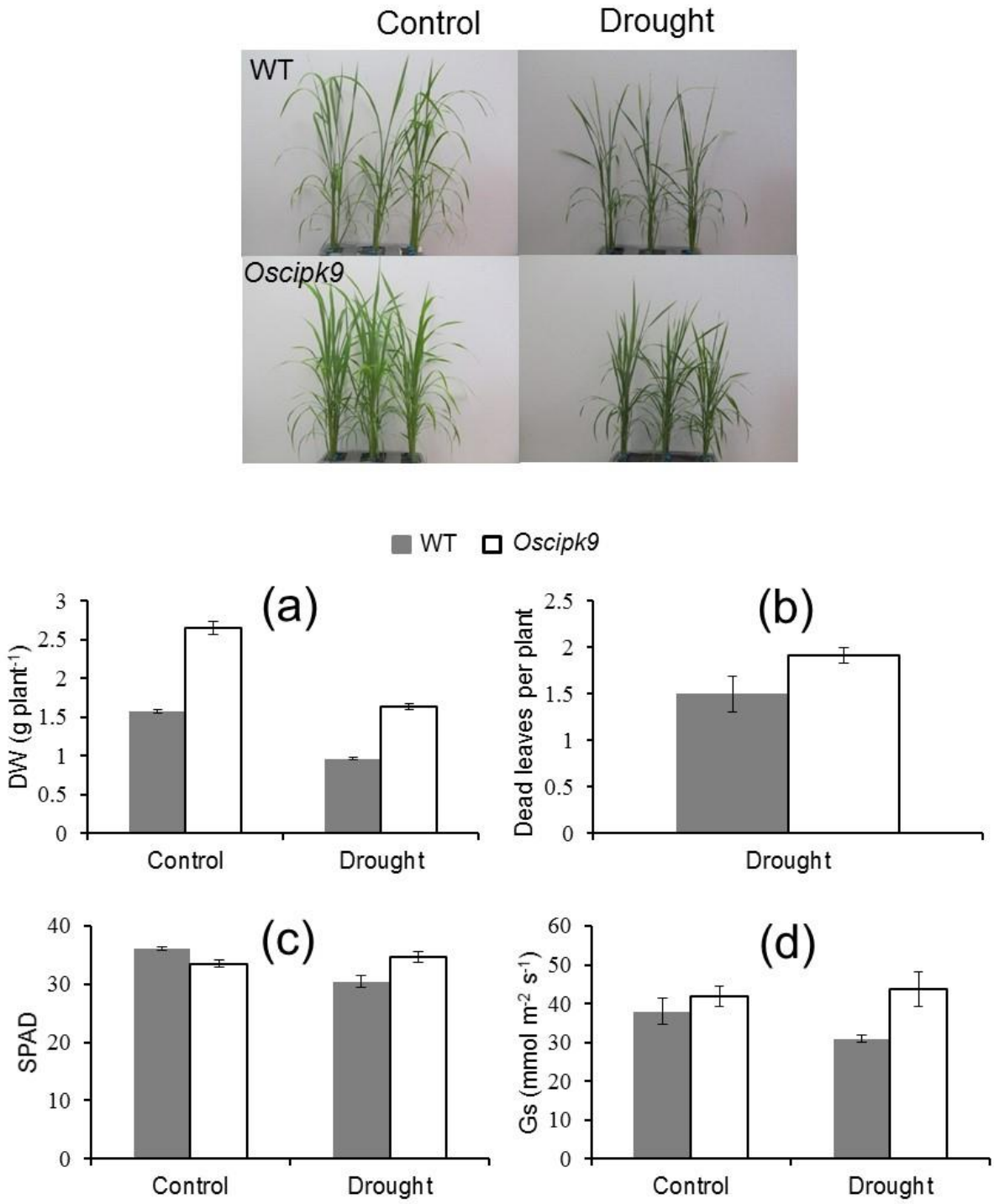

Figure 2. 4 Effect of drought stress by PEG (4000) on the growth of the *Oscipk9* mutant plants and the WT: (a) dry weight, (b) number of dead leaves per plant, (c) chlorophyll content (SPAD value), and (d) Stomatal conductance. The plants of both lines were grown in a hydroponic system for 21 days. The WT (dark bars), *Oscipk9* mutant line (white bars). Data are the mean ± SE (n=18).

A lower shoot K^+: Na^+ ratio was recorded in the *Oscipk9* mutant compared to the WT under the control condition. While drought stress resulted in a significant decrease in the shoot

Na^+ content of the *Oscipk9* mutant (Fig. 2.5a), it had no significant effect on its shoot K^+ content (Fig. 2.5c). However, both the shoot Na^+ and K^+ content increased significantly by 44%, and 70%, respectively in the WT line compared to the control (Fig. 2.5a, c). The shoot osmolality of both lines was affected equally by drought stress, increasing by 45% (Fig. 2.5e).

The roots K^+: Na^+ ratio was lower in the *Oscipk9* mutant than in the WT by 25% under the drought condition. While this condition had the same effect on the root Na^+ content in both lines, reducing it by 40-50% (Fig. 2.5b), its effect was much more pronounced on the root K^+ content of the *Oscipk9* mutant, where it decreased by 26%. However, it had no effect on the root K^+ content of the WT (Fig. 2.5b). In contrast, root osmolality underwent a slight increase under the drought stress condition, but with no significant difference between the *Oscipk9* mutant and the WT (Fig. 2.5f). It is worth noting that the control plants in the salinity experiment have only half as much root K^+ as the control grown plants in drought conditions. This difference could be due to any of the considerable number of factors affecting plant growth that the plants were exposed to as the two experiments were conducted separately. For example, the salt experiment was carried out between November and January, while the drought one was conducted between February and March, thereby making weather one of the factor.

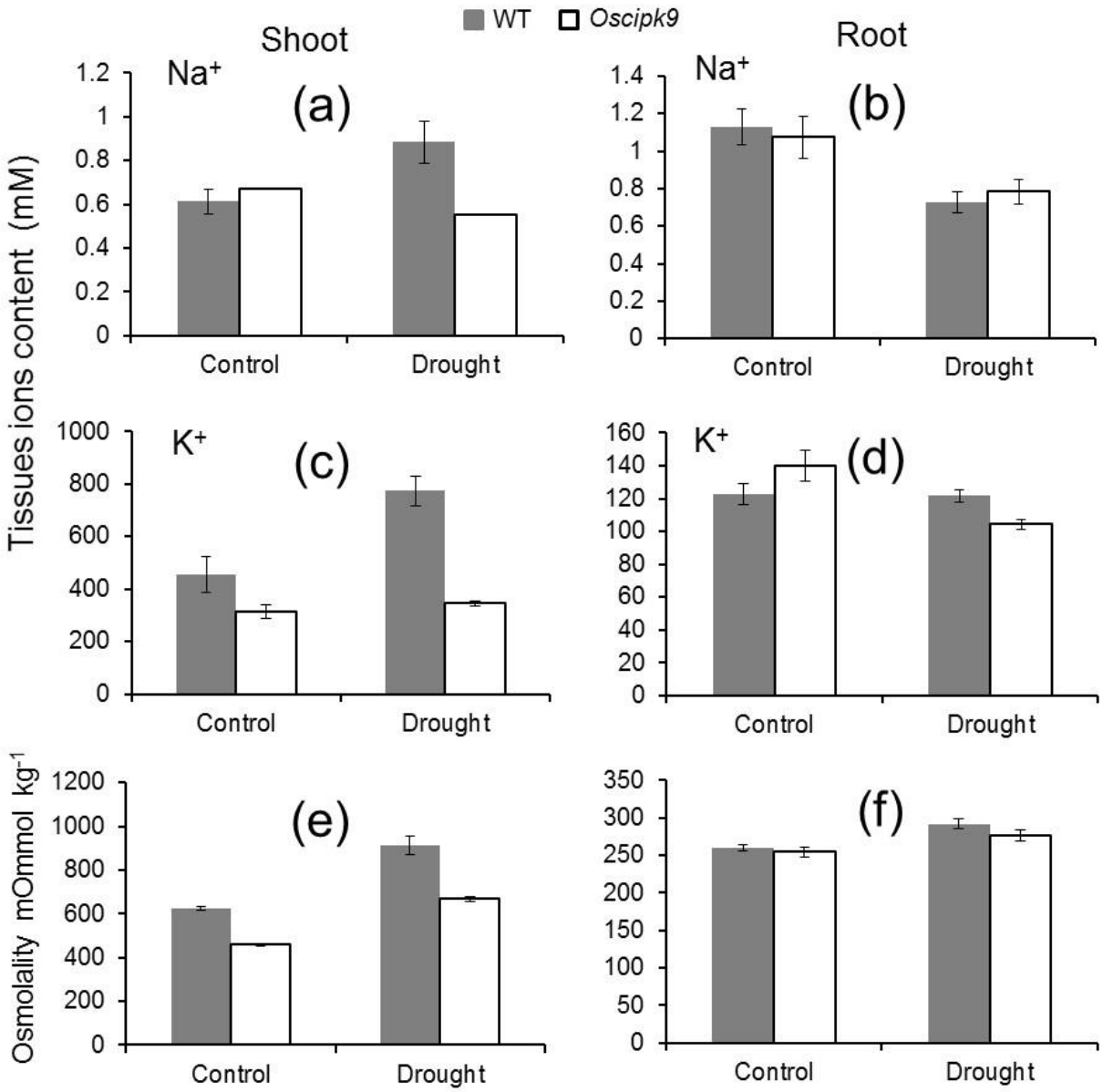

Figure 2. 5 Effect of drought stress both on shoot and root K^+ and Na^+ contents and osmolality of *Oscipk9* mutant plants compared to the WT. (a) shoot Na^+ content, (b) root Na^+ content, (c) shoot K^+ content, (d) root K^+ content, (e) shoot osmolality, and (f) root osmolality. The plants of both lines were grown in a hydroponic system for 21 days. The WT (dark bars), *Oscipk9* mutant line (white bars). Data are the mean ± SE (n=6).

2.3.3 Plant growth under varying concentrations of K^+ and Ca^{2+}

While there was no difference between the dry weights of both lines, when they were grown in control, and 0.1 Ca^{2+} conditions, the *Oscipk9* mutant experienced inhibited growth and accumulated a significantly ($P \leq 0.01$) lower dry weight under the high Ca^{2+} and K^+ deficiency

conditions (Fig. 2.6a). In contrast, under the high K^+ condition, the *Oscipk9* mutant had a higher dry weight compared to the WT (Fig. 2.6a). It should be noted that the growth of plants in the K^+ deficient concentration (0.1 mM) was superior to that of the control plants, which were cultivated in a normal Hoagland solution, as detailed in the Growth Conditions Section (2.2.1. However, there are many factors affecting plant growth that are beyond our control. Since the aim of this study is to identify the role of CIPK9 in rice plants by studying control and mutant lines, the comparison should be made between the wild type and the mutant, under the same conditions. It is clear that there was no significant difference between them under control condition, but the wild type plants did perform better, in terms of growth, when compared to the mutant plants under K^+-deficient condition.

The greatest difference in stomatal conductance (Gs) was observed between both lines, under the control, and Ca^{2+} conditions, where it ranged between 37-53% (Fig. 2.6b). However, this difference decreased by 21%, under the high K^+ condition, and was negligible, under the K^+ deficiency condition (Fig. 2.6b).

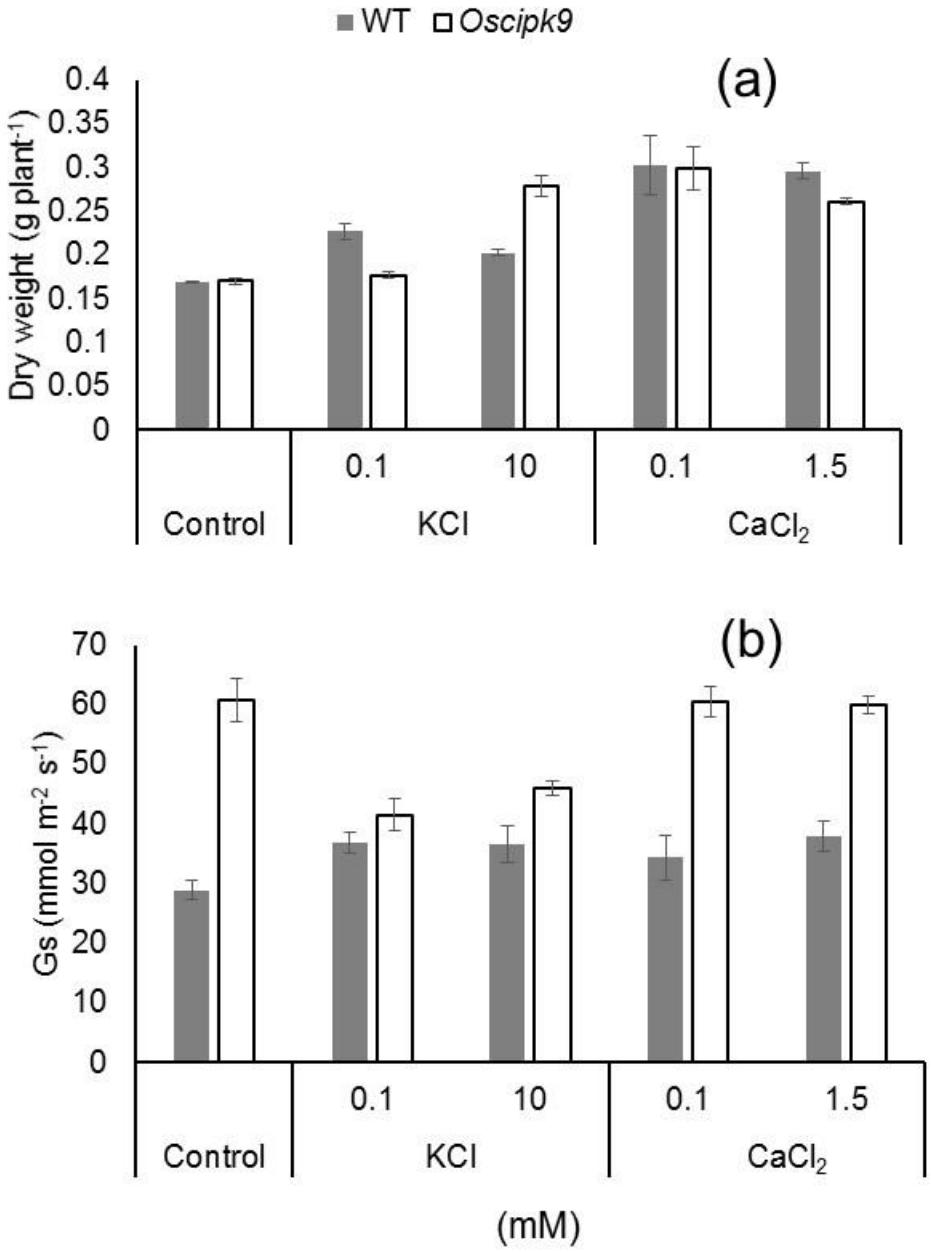

Figure 2. 6 Effects of two levels of calcium (0,1 and 1.5 mM of $CaCl_2$), and two levels of potassium (0.1 and 10 mM of KCl) on the (a) dry weight and (b) stomatal conductance of *Oscipk9* mutant plants and the WT. Plants of both lines were grown under these conditions in a hydroponic system for 3 weeks. The WT (dark bars), *Oscipk9* mutant line (white bars). Data are the mean ± SE (n=18).

Although the *Oscipk9* mutant showed a significantly lower shoot Na^+ and K^+ content than the WT under the control condition (Fig. 2.7a, c), the shoot K^+: Na^+ ratios of both lines were identical. Furthermore, this ratio was significantly reduced by 28-38% in the *Oscipk9* mutant compared to the WT under varying Ca^{2+} and K^+ conditions (Fig. 2.7a, c). Shoot osmolality was

also significantly lower in the *Oscipk9* mutant compared to the WT (Fig. 2.7e). However, the root K^+: Na^+ ratio was significantly higher in *Oscipk9* mutant compared to the WT under all conditions (Fig. 2.7b, d). Under the K^+ deficiency condition, the lowest root K^+: Na^+ ratio was recorded in the WT, followed by in the *Oscipk9* mutant. This was due to a significant reduction in the root K^+ content of the WT accompanied by a significant increase in the root Na^+ content of both lines.

It is worth mentioning that the increase in t root Na^+ content was probably the result of supplementing the medium with 1.875 mM Na^+, because all potassium salts in the control medium were replaced with equimolar quantities of the corresponding sodium salts to create the low K^+ as mentioned in Section 2.2.2 (Treatments). Meanwhile, root osmolality was the same both in the *Oscipk9* mutant and the WT (Fig. 2.7f).

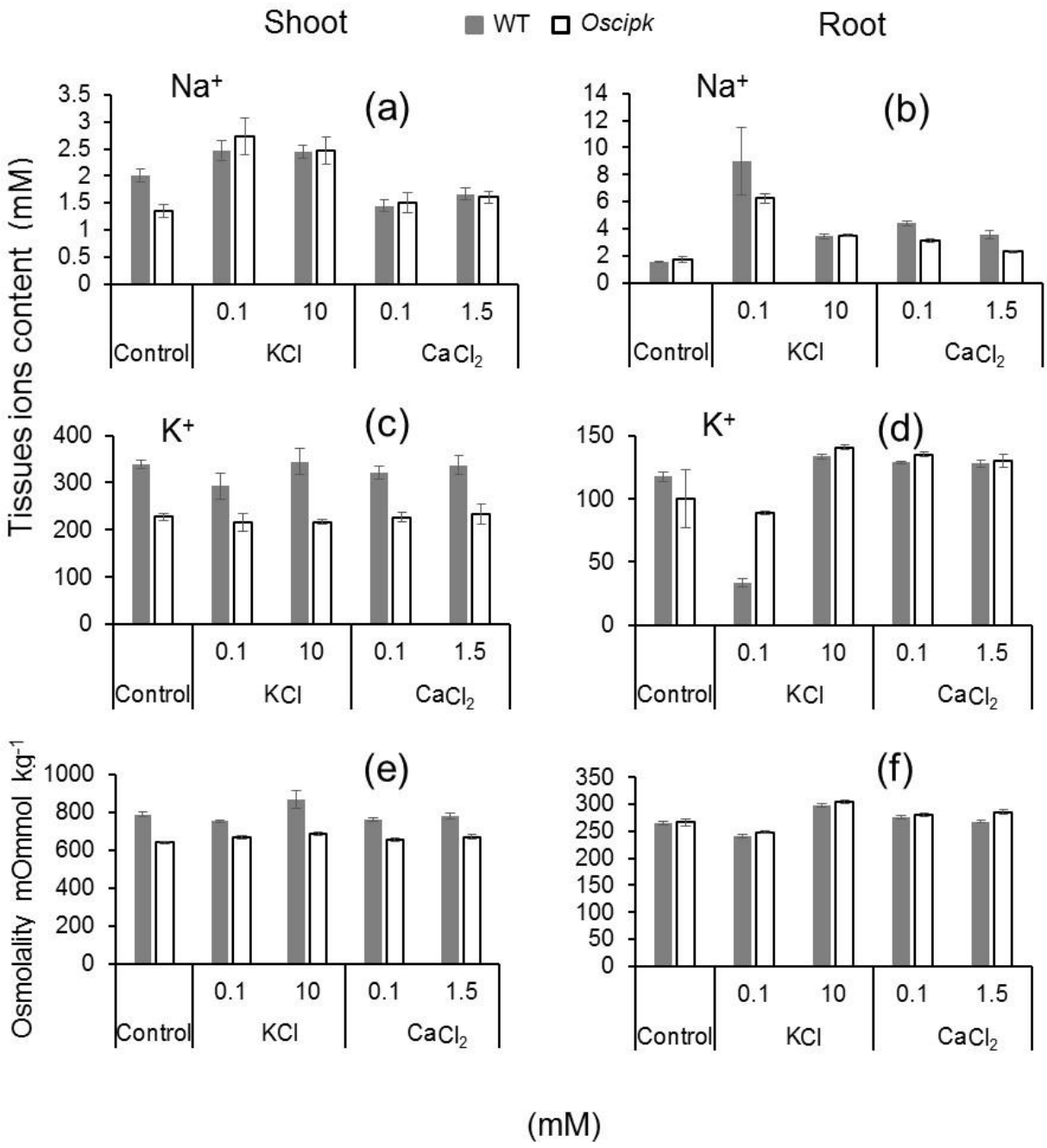

Figure 2. 7 Effect of two levels of calcium (0,1 and 1.5 mM of $CaCl_2$), and two levels of potassium (0.1 and 10 mM of KCl) on the (a) shoot Na^+ content, (b) root Na^+ content, (c) shoot K^+ content, (d) root K^+ content, (e) shoot osmolality, and (f) root osmolality of the *Oscipk9* mutant plants and the WT compared to those under the control condition. Plants of both lines were grown under these conditions in a hydroponic system for 3 weeks. The WT (dark bars), *Oscipk9* mutant line (white bars). Data are the mean ± SE (n=6).

2.3.4 Root ion fluxes responses to oxidative stress

Since ROS generation is one of the most common plant responses to abiotic stresses, such as salt and drought (Demidchik, 2010), this study used only H_2O_2 to trigger the gene expression and investigate the function of this expression via direct oxidative stress.

The finding of this study showed that both under the high Ca^{2+} and low K^+ conditions, adding 5 mM H_2O_2 to the measuring medium caused an immediate K^+ efflux, which was significantly higher by 100% in the *Oscipk9* mutant compared to the WT (Fig. 2.8a). Over the time, K^+ efflux continued to decrease, reaching its lowest levels (-90 and -55 nmol m^{-2} s^{-1} in the *Oscipk9* mutant, and WT, respectively) after 8 minutes of treatment. After this, the level remained constant until the end of the measurement phase (Fig. 2.8a). In addition, when the rice plants were grown under the low Ca^{2+}/low K^+ condition, the effect of the H_2O_2 was much greater on the *Oscipk9,* causing a higher K^+ efflux (Fig. 2.8b). This efflux peaked in the *Oscipk9* mutant at -155 nmol m^{-2} s^{-1}, while in WT the level of K^+ flux was similar to that under a high Ca^{2+}/low K^+ condition (Fig. 2.8b). Under the same condition (low Ca^{2+}/low K^+), the K^+ efflux was accompanied by a gradual increase in Ca^{2+} influx in both lines. This reached a peak of 25 and 17 nmol m^{-2} s^{-1} for the *Oscipk9,* and WT, respectively, after 8 min of treatment, and then, started to decrease (Fig. 2.9b). In contrast, both lines showed K^+ influx when grown in a high K^+ and either a high or low Ca^{2+} condition (Fig. 2.8c, d), where adding H_2O_2 caused an immediate K^+ influx that reached 600 nmol m^{-2} s^{-1} and then gradually deceased (Fig. 2.8c, d).

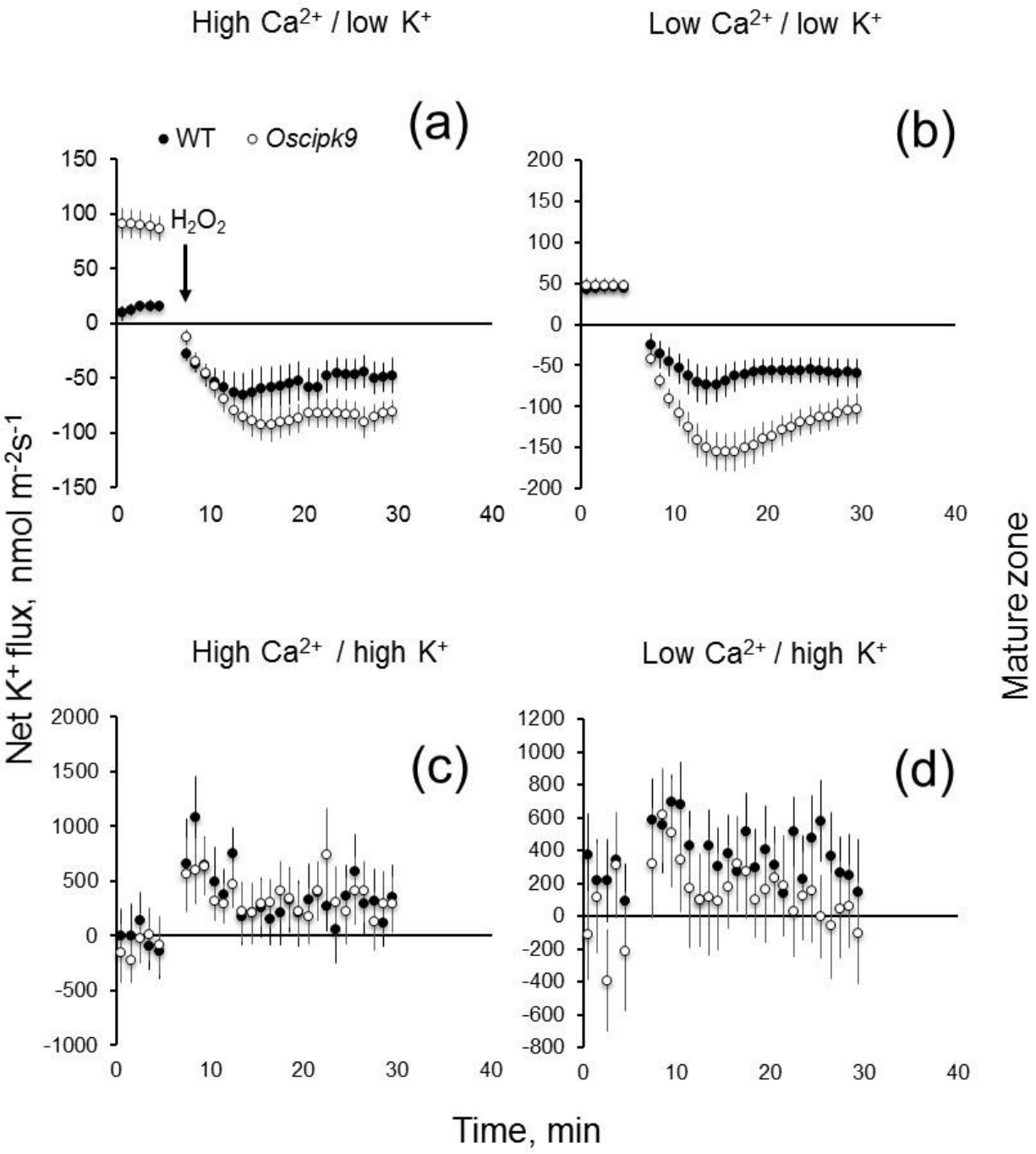

Figure 2. 8 The H_2O_2 induced K^+ efflux in the mature zone of *Oscipk9* mutant plants and the roots of the WT when these plants were grown under a low K^+ condition and either low/ high Ca^{2+} condition (a, b), while H_2O_2 induced K^+ influx in the same zone of *Oscipk9* mutant plants and the WT when these plants were grown under a high K^+ condition and under either a low/ high Ca^{2+} condition (c, d). 5-6-day-old seedlings were treated with 5 mM of H_2O_2. Data are the mean ± SE (n=6)

The addition of H_2O_2 caused a significant increase in Ca^{2+} influx, under the low Ca^{2+}/low K^+ condition, which was much more pronounced in the *Oscipk9* mutant than in the WT (Fig. 2.9b). Under the other conditions (high Ca^{2+}/ low K^+, high Ca^{2+}/high K^+, and low Ca^{2+}/high K^+), no significant difference was found between the *Oscipk9* mutant and WT in response to the H_2O_2 treatment (Fig. 2.9a, c, d). However, the lowest Ca^{2+} influx was recorded under the low Ca^{2+}/ high K^+ condition (Fig. 2.9d). Initially, this was Ca^{2+} efflux which turned into Ca^{2+} influx by 0.6-1.68 nmol m^{-2} s^{-1} after 8 minutes of H_2O_2 treatment (Fig. 2.9d), with the highest influx being recorded under the high Ca^{2+}/ low K^+ condition (Fig. 2.9a).

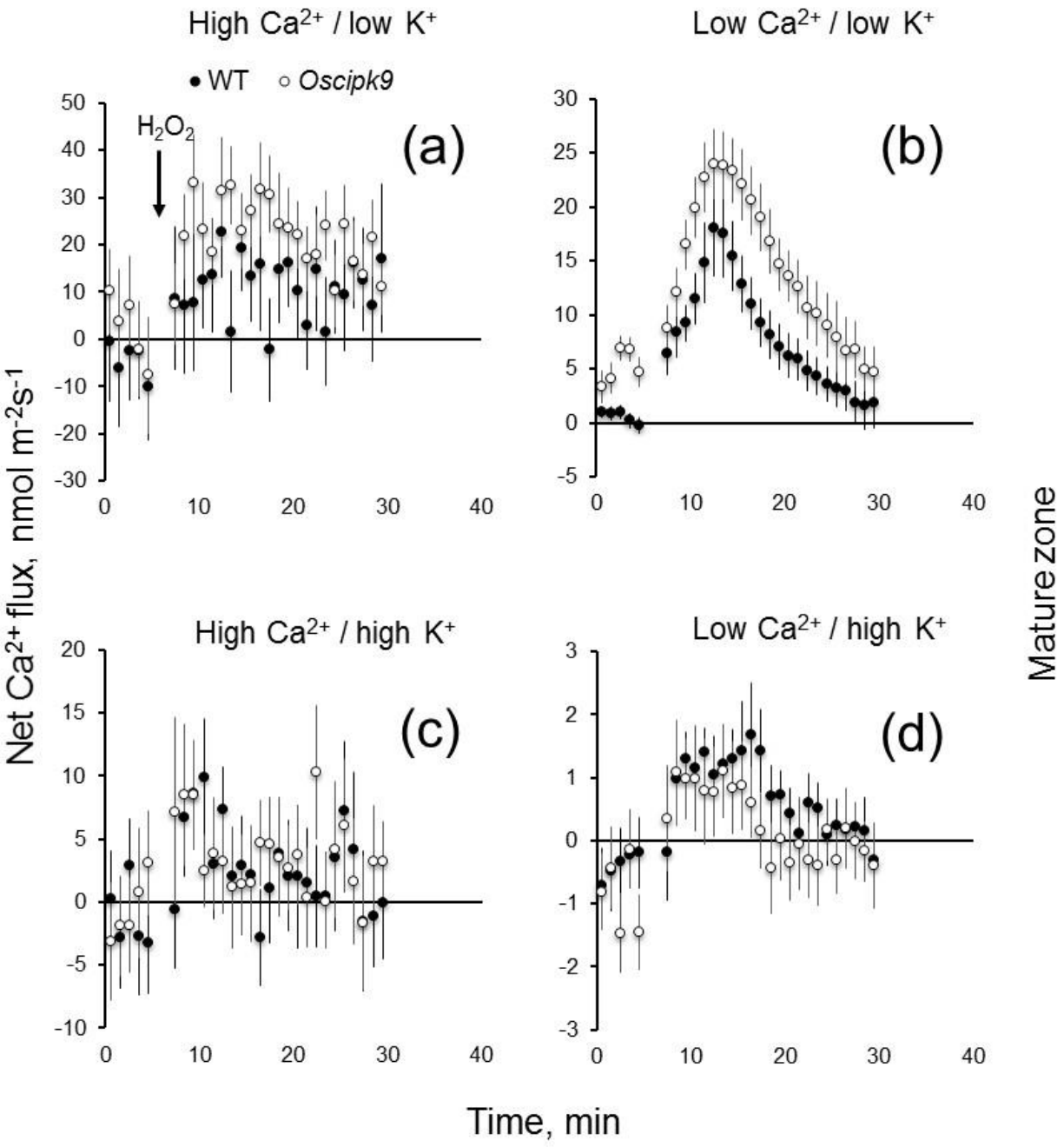

Figure 2. 9 H_2O_2 induced Ca^{2+} efflux in the mature zone of *Oscipk9* mutant plants and the roots of the WT when these plants were grown under a low K^+ condition and either low/ high Ca^{2+} condition (a, b), while H_2O_2 induced K^+ influx in the same zone of *Oscipk9* mutant plants and the WT when these plants were grown under a high K^+ condition and under either a low/ high Ca^{2+} condition (c, d). 5-6-day-old seedlings were treated with 5 mM of H_2O_2. Data are the mean ± SE (n=6).

2.4 Discussion

Salinity, drought, and K^+ deficiency have a negative impact on most of the morphological, physiological and biochemical processes that influence plant metabolism and growth in many glycophyte crops including rice. However, despite the key molecular mechanisms by means of which plants perceive, transduce, and regulate gene expression to adapt to abiotic stresses, having been the focus of numerous studies over recent years, they are still not well understood. This chapter investigates the role of CIPK9 as a component of this adaptive mechanism in rice exposed to salinity, drought, and K^+-deficientt conditions.

2.4.1 Loss of function of *Oscipk9* increased the root K^+ efflux under the low-K^+ condition, and Ca^{2+} deficiency caused a further K^+ efflux when oxidative stress induced

Salinity, drought (Miller et al., 2008; Mittler, 2002)) and K^+ deficiency (Shin and Schachtman, 2004) conditions induce accumulation of reactive oxygen species (ROS) in a dose-dependent manner (Bhattacharjee, 2012). Several studies suggest that ROS, in particular H_2O_2 is signal transduction molecule, which is involved in mediating responses to environmental stresses and developmental signals, under normal conditions (del Río et al., 2006; Miller et al., 2008; Miller et al., 2010). In a high concentration, ROS causes cellular damage, or hypersensitivity, and eventually, programmed cell death (Mittler et al., 2004). Another well-known intracellular second messenger molecule, which is involved in many signal transduction pathways in plants, is the Ca^{2+} ion. Furthermore, it is involved in the maintenance of the K^+:Na^+ ratio, as well as ion selectivity, under salt stress (Kader and Lindberg, 2010; Tuteja, 2009). An increase in the cytosolic Ca^{2+} in plant cells has been linked to their responses to various environmental stresses, including salt, drought (Tuteja and Mahajan, 2007) and ROS activation (McAinsh et al., 1996; Mori and Schroeder, 2004; Pei et al., 2000). This increase, which is transient can be detected by a number of calcium sensors, and a critical signal to stimulate the plants' physiological responses to abiotic stresses (Tuteja and Mahajan, 2007). One of these sensors, is a calcineurin B-like protein (CBL)-

CBL- interacting protein kinase (CIPK) complex, which transduces a signal downstream by modulating transcription factors, transporters channels or pumps in response to different abiotic stresses (Manik et al., 2015), resulting in the expression of a number of oxidative stress-induced genes. The MIFE data in this study provides evidence of a potential molecular connection between the activity of CBL-CIPKs complex, which may be stimulated by the H_2O_2-induced calcium signal, and the activity of K^+ transporters in the plasma membrane of the epidermal cells of root. However, The *OsCIPK9* gene expression should be checked in order to confirm the assumption that the *OsCIPK9* expression increased in response to oxidative stress and, so, regulates the K^+ transporters or channels.

As shown in (Fig. 2.8a, b), H_2O_2 application caused a transient K^+ efflux. This may be mediated both by GORK channels (Demidchik et al., 2010) and non-selective cation channels (NSCCs) (Pottosin et al., 2012) under the low K^+ condition, while K^+ influx was recorded under the high K^+ condition (Fig. 2.8c, d). Additionally, under all conditions, K^+ flux was accompanied by Ca^{2+} influx (Fig. 2.9a, b, c, d), which can be mediated by NSCCs (Demidchik and Maathuis, 2007). These H_2O_2-induced K^+ efflux and Ca^{2+} influx can be divided into three different observation based on the concentration of the Ca^{2+} and K^+ in the media in which the plants were grown, prior to ion flux. The first one is comprised of plants grown in a high Ca^{2+}/low K^+ condition, a condition that makes the high-affinity K^+ transport systems active. Under this condition, and when hydrogen peroxide (H_2O_2) was applied, the *Oscipk9* mutant had 100% higher K^+ efflux (Fig. 2.8a) accompanied with a relatively higher Ca^{2+} influx (Fig. 2.9a) compared to the WT. These results suggest that H_2O_2 might induced Ca^{2+} influx, which in turn generates what is known as a Ca^{2+} signature. This signature was detected in the WT by the CBL-CIPK9 complex, which then produced a signal which may have regulated the activity of the K^+ transporters responsible for the transport of K^+ across the plasma membrane. To test this assumption, the Ca^{2+} signal would require some type of aequorin expressing transgenic plants, as well as a way of

measuring luminescence within seconds of applying H_2O_2. One suggestion would be to transform the wild type and*Oscipk9* rice plants with an aequorin gene, and then apply H_2O_2 to the roots of those plants to see if whether the Ca^{2+} signal was weaker or abolished in the mutants.

However, in the *Oscipk9* mutant, loss of function of the *cipk9* meant that CBL-CIPK9 complex could not form; it is this complex that is required to intercept the signal from the Ca^{2+} signature to modulate the activity of K^+ transporter proteins. Therefore, the K^+ efflux was higher in the *Oscipk9* mutant compared to the WT. It is worth taking into consideration the fact that, as rice plants have 30 CIPKs, some of these proteins are regulating the activity of K^+ transporters, as it is noted in the literature review. Therefore, these might be compensating of the loss of *OsCIPK9* function in response to abiotic stress, a possibility that needs to be investigated in a future study on the expression of other *CIPKs* genes.

An earlier study showed that mutation in the NADPH oxidase *rhd2* mutant inhibits ROS formation, which in turn causes Ca^{2+} deficiency (Foreman et al., 2003). Consequently, the *rhd2* mutant had short root hairs and stunted roots, while treating the *rhd2* root with ROS stimulated the activity of plasma membrane hyperpolarisation, which then activated Ca^{2+} channels. This suggested that the ROS-activated Ca^{2+} channel may have facilitated Ca^{2+} influx for cell elongation in different root cell types (Foreman et al., 2003). The second observation in this study is stronger K^+ efflux that was observed, when the plants were grown under the low Ca^{2+}/low K^+ condition. This efflux increased by 100% in the *Oscipk9* mutant, but did not change in the WT compared to previous condition (high Ca^{2+}/ low K^+) (Fig. 2.8b). These results suggest that exogenous Ca^{2+} may have an ameliorative effect on H_2O_2-induced K^+ efflux from roots, by rapidly increasing cytosolic Ca^{2+} to generate a signal which can regulate the activity of K^+ transporters. These results are consistent with those of (Shabala et al., 2006b) who investigated the effect of extracellular Ca^{2+} on NaCl-induced K^+ efflux. Their results showed that Na^+-induced K^+ efflux was mediated by two groups of outwardly directed K^+ permeable channels: depolarisation-activated K^+ channels

(DAPCs) and non-selective cation channels (NSCCs). An increase in extracellular Ca^{2+} regulates both these channels and prevents K^+ efflux from roots. The third observation in this study is K^+ influx, which occurred when the plants were grown in the high K^+ and either high or low Ca^{2+} conditions (low affinity K^+ transport systems) resulting in a similar K^+ influx in both lines. This indicates that the K^+ concentration is a key determinant for CIPK function (Fig. 2.8c, d), where high K^+ concentration is needed to stimulate the activity of low-affinity K^+ uptake transporters in the root. This activity may have masked the loss of function of *Oscipk9* that induced further K^+ efflux, which could have been present a very low rate. However, previous studies found that CIPK9 did not interact with any potassium transporters, either in the yeast two-hybrid system or Arabidopsis including AKT1, HAK5, AKT2, SKOR, and TPK1 (Liu et al., 2013; Pandey et al., 2007). It has also been reported that high CIPK9 expression was induced when Arabidopsis plants were grown, under a K^+-deficient condition (Pandey et al., 2007). The result was that two *cipk9-1* and *cipk9-2* mutants in particular became hypersensitive and showed impaired growth. These results suggested that a low K^+ condition generates H_2O_2 and this regulates the activity of CIPK through a Ca^{2+} signature-CBL interaction. CIPK9 activity is required for the K^+ transporters involved in K^+ homeostasis (Pandey et al., 2007). Under a low-K^+ condition, CIPK23, which is closely related to CIPK9, regulates the activity of AKT1, thereby enhancing the K^+ uptake and increasing K^+ content in Arabidopsis and rice plants (Li et al., 2014; Xu et al., 2006). A very recent study investigating the role of CIPK3, 9, 23 in Mg^{2+} homeostasis in Arabidopsis showed that the *cbl2*, *cbl3* double mutant was extremely sensitive to external Mg^{2+} under low Ca^{2+} conditions. Furthermore, Ca^{2+} uptake was inhibited by the presence of a high Mg^{2+} concentration in the medium, suggesting that this alters the cytosolic Ca^{2+} concentration, causing what it is known as a Ca^{2+} signature. This may then be encoded by CBL2 and CBL3 to regulate the downstream protein kinase CIPK3, 9, and 23. This Ca^{2+} signature-CBL-CIPK complex regulates the activity of proteins that transport Mg^{2+} into the vacuoles, protecting the plant from toxic levels of Mg^{2+} (Tang et al.,

2015). However, the role of OsCIPK9 in Mg^{2+} transport in rice plants needs to be investigated in the future studies.

2.4.2 The *Oscipk9* mutant maintained a lower K^+: Na^+ ratio than the WT under abiotic stress conditions

A plant's ability to maintain a high K^+: Na^+ ratio is considered to be a key determinant of its salt tolerance (Maathuis and Amtmann, 1999). In the present study, at the shoot level, the *Oscipk9* mutant maintained a lower K^+:Na^+ ratio than the WT both under the control and salt stress conditions (data are calculated based on the data presented in Fig. 2.3). This was not due to any significant difference in the Na^+ content, but resulted from lower K^+ content both under the control and salt conditions (Fig. 2.3). These results suggest that OsCIPK9 functioned specifically in K^+ homeostasis and was not involved in shoot Na^+ homeostasis. At the root level, although the *Oscipk9* mutant had a higher K^+ content, it maintained a lower K^+:Na^+ ratio because of its significantly higher root Na^+ content. Several studies noted that *CIPK9* expression was triggered under salt stress (Das and Pandey, 2010; Li et al., 2009). For example, *CIPK* mRNA levels rapidly increased after 1 h of salt treatment, reaching a maximum at 3h, followed by a decline (Pandey et al., 2007). However, there has been no evidence to suggest that CIPK9 plays an important role in Na^+ homeostasis.

Plant growth is adversely affected by water stress. The subjection of plants to long-term water stress can restrict K^+ influx, thus lowering tissue K^+ content, which can further impair plant growth. High K^+ uptake is, therefore, critical for plant drought tolerance. Osmotic adjustment is an important mechanism used by plants to maintain high turgor pressure in response to drought stress. Since inorganic osmotica, such as K^+, and Na^+ play an important role in osmotic adjustment, an increase in K^+ content, which is mediated by plasma membrane voltage-gated K^+ transporters, would significantly lower osmotic potential, thereby helping the plant to maintain a high cellular turgor potential under water-stress conditions. The results of the drought stress experiment

conducted by the present study showed that the *Oscipk9* mutant maintained a lower shoot K^+: Na^+ ratio in shoots and roots both under the control and drought stress conditions, (Fig. 2.5a, c). Upon exposure to drought stress, the shoot K^+, Na^+ content of the WT increased significantly, with no corresponding effect on the shoot K^+ content of the *Oscipk9* mutant (Fig. 2.5a, c). However, the root K^+ content was reduced by drought stress in the *Oscipk9* mutant (Fig. 2.5d) but not in WT. These results suggest that the WT, which possesses a normal *CIPK* expression level, managed to maintain a high osmotic potential by accumulating more inorganic osmotica K^+ and Na^+. This points to OsCIPK9's playing a direct role in the K^+ homeostasis that was needed for osmotic adjustment under water stress. A study on Arabidopsis plants, showed that *CIPK9* mRNA increased rapidly after 1 h of mannitol treatment, and attained a maximum at 6 and 12 h and then reduced at the 24 h point (Pandey et al., 2007). In rice, *CIPK9* expression was induced when the plants were exposed to drought stress (Xiang et al., 2007).

Since, the *Oscipk9* mutant displayed the same behavior by maintaining a lower K^+: Na^+ ratio compared to the WT under varying K^+, and Ca^{2+} conditions (Fig. 2.7), it may play a key role in K^+ homeostasis. It is notable that the shoot K^+ content did not change in the *Oscipk9* mutant, indicating that this mutant does not directly affect K^+ accumulation in shoot tissues. This result is in line with the findings of previous studies, which revealed that under the low K^+ condition, neither K^+ content nor uptake was changed in the *Oscipk9* mutant (Pandey et al., 2007). This indicated that the hypersensitivity in the phenotype was caused by a low K^+ content under low K^+ condition. The suggestion from this is that the downstream targets for CIPK9 may not be directly related to K^+ uptake (Pandey et al., 2007).

2.4.3 *Oscipk9* mutant displayed lower plant growth and stomatal conductance under the low K^+ condition but not in response to other abiotic stresses

The results indicated that there were no significant phenotype changes in the *Oscipk9* mutant compared to the WT under the salt, drought, and varying Ca^{2+} conditions (Fig. 2.2, 2.4,

and 2.6). Interestingly, the only phenotypic difference between the *Oscipk9* mutant and the WT was found when both were grown under a low K^+ condition for 21 days: the *Oscipk9* had a lower dry weight compared to the WT (Fig. 2.6a). Furthermore, stomatal conductance significantly reduced in the *Oscipk9* mutant, under the low K^+ condition compared to the control condition (Fig. 2.6b). These results demonstrated that the low K^+ sensitivity in the phenotype of the *Oscipk9* mutant was caused by a disruption in the function of OsCIPK9. This suggested that the OsCIPK9 specifically regulated K^+ homeostasis, in rice under low K^+ stress. A study on the role of CIPK9 in low potassium tolerance in Arabidopsis showed that when the plants were grown in a low potassium medium, the growth of the *Oscipk9* mutant plants was significantly inhibited compared to the WT. On the other hand, no phenotypic difference was evident between the *cipk9* mutant and WT under cold, saline, ABA, auxin, or cytokinin conditions (Pandey et al., 2007). In contrast, other studies, which investigated the effect of *CIPK9* overexpression on the growth of Arabidopsis, showed that the overexpressed *CIPK9* accumulated less K^+ compared to the WT when the plants were grown, under the low K^+ condition. This suggestted that *CIPK9* expression is negatively correlated with low K^+ tolerance in plants (Liu et al., 2013).

Overall, the finding of this study revealed that the calcineurin B-like protein-interacting protein kinase 9 (CIPK9) played a critical regulatory role in K^+ homeostasis in rice plants, particularly under the K^+-deficient condition. A higher K^+ efflux was observed in the root of *Oscipk9* mutant plants grown under a low Ca^{2+} and K^+ condition compared to the wild type in response to oxidative treatment. When the external Ca^{2+} concentration increased in the growth medium, the K^+ efflux was reduced in response to the oxidative stress. Meanwhile, no significant difference in K^+ flux was observed between the lines when the rice plants were grown in a medium containing a high K^+ concentration.

Chapter 3

The role of the high-affinity potassium transporters OsHAK1 and OsHAK5 in the salt tolerance of rice plants grown in potassium deficient soils

3.1 Introduction

Potassium plays a crucial role in plant biochemical and biophysical processes, including photosynthesis, stomata movement, enzyme activation, and osmotic adjustment (Szczerba et al., 2009), and hence, also in plant growth and development. However, the mechanisms of uptake and translocation of K^+ in plants are known to be negatively affected by environmental stresses, such as salinity, drought and K^+-deficiency (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008). Therefore, plants have evolved a complex network of potassium transporters and transport regulators to maintain a functional cytoplasmic K^+ concentration, which is a prerequisite for plant growth and development, particularly under adverse environmental conditions.

Epstein et al. (1963) were the first to observe the biphasic kinetic uptake of potassium. The first of these mechanisms, which requires an external K^+ concentration less than 1 mM K, is referred to as the high-affinity transport system (HATS). This mechanism uses transporters to mediate K^+ influx, a process that is energy-intensive (Maathuis and Sanders, 1996). This is because acute depolarisation of the plasma membrane occurs when the K^+ ion is transported into the cytoplasm and retained there. Thus, for the membrane to regain its potential, an energy–consuming mechanism, involving H^+-ATPase is needed to pump one proton out of the plant cell (Britto and Kronzucker, 2008). This H^+-ATPase regulates the K^+ influx by creating a H^+ gradient that energises the co-transporter-mediated high-affinity K^+ uptake (Shabala and Pottosin, 2014).

The second mechanism is referred to as the low-affinity transport system (LATS), a mechanism which exhibits a relatively low K^+ selectivity compared to that of other alkali cations

(Maathuis and Sanders, 1996). In this case, K^+ influx is mediated by ion channels implying that the thermodynamically passive movement of the K^+ ion, along the electrochemical gradient of K^+ is most likely determined by inter/extra cellular K^+ concentrations and plasma membrane potential (Britto and Kronzucker, 2008).

The function of each system relies on the external K^+ concentration. HATS is significantly down-regulated under K^+-sufficient conditions and, inversely, is significantly up-regulated under K^+-deficient conditions (Britto and Kronzucker, 2008). In contrast, LATS-mediated K^+ influx is up-regulated by an increase in the external K^+ concentration (Britto and Kronzucker, 2008).

HATS and LATS also differ in the way K^+ uptake is affected by the presence of other ions. With HATS, K^+ uptake is very sensitive to monovalent Na^+ and NH_4^+ cations, while with LATS, it is relatively resistant to their influence (Maathuis et al., 1997; Maathuis and Sanders, 1996; Nieves-Cordones et al., 2008).

Yet another difference is that the permeability of the plasma membrane is much higher in the low-affinity transport system, due to a high efflux-to-influx ratio, than in the HATS (Britto and Kronzucker, 2008). The reason for this is that K^+ efflux can occur as a result of physical disturbance, caused by the engagement of mechanically or stretch-activated channels (Shabala et al., 2000). Therefore, because of the higher ratio of K^+ efflux-to-influx observed in the LAST range, steady state influx measurements taken under a high external K^+ concentration are likely to be less accurate than those obtained under HATS conditions, and so result in inaccurate K^+ influx measurements (Szczerba et al., 2006).

Potassium is the key to regulating the plasma membrane potential in plant cells. Plants evolve different voltage gated channels to equilibrate membrane potential in response to environmental stimuli. For example, membrane depolarisation that occurs in response to a high external K^+ concentration, or the addition of NH_4^+, results in K^+ efflux which is mediated via outward-rectifying K^+ channels. On the other hand, K^+ deficiency causes a decrease in the

equilibrium potential for K^+, resulting in membrane hyperpolarization (Wang and Wu, 2013). This in turn, increases K^+ influx via inward rectifying channels (Britto and Kronzucker, 2008).

It has been reported that the majority of K^+ flux is mediated by the activity of transporters of the *KT/HAK/KUP* family in a high-affinity transport system (Britto and Kronzucker, 2008). The loss of function of *Athak5*, a member of *KT/HAK/KUP* family, significantly reduces K^+ net uptake in HATS ranges, thereby causing a reduction in the fresh tissue weight (Nieves-Cordones et al., 2010). This suggests that AtHAK5 plays a crucial role in K^+ acquisition, particularly under HATS ranges. Moreover, while *AtHAK5* expression was up-regulated by 6-fold under HATS conditions, it was down-regulated, even under HATS, with the addition of NaCl to the growth medium, this is due to membrane depolarization (Nieves-Cordones et al., 2010). In terms of transport regulators, a study has revealed that interaction, CBL1-CIPK23, up-regulates the activity of *AtHAK5*. This occurs because phosphorylation of the N terminus of *HAK5* increases the affinity of K^+ uptake in the root of Arabidopsis under high-affinity potassium conditions (Ragel et al., 2015).

In the rice genome, 27 genes of the *KT/HAK/KUP* family have been identified (Yang et al., 2009), of which *OsHAK1* and *OsHAK5* are thought to be the most vital in mediating K^+ uptake, under HATS (Yang et al., 2014). A number of studies have described the physiological role of OsHAK1 and OsHAK5 in K^+ homeostasis, in the various rice genotypes, under varying environmental conditions. For example, in a study by Yang et al. (2014) overexpression of *OsHAK5* increased plant salt tolerance, by causing a rise in the shoot K^+:Na^+ ratio, under low potassium conditions. An opposite result was obtained when *OsHAK5* was knocked out. This suggested that OsHAK5 plays a key role in the acquisition of K^+, via the high-affinity transport system.

In a study by Chen et al. (2015) the expression of *OsHAK1*, another member of HATS, was found to be up-regulated in various plant tissues, particularly in the root and shoot meristem, under both K^+-deficiency and salt stress. Loss of function of *Oshak1* resulted in a significantly

reduced acquisition of K^+, which caused stunted roots and shoots, and eventually growth impairment. Moreover, loss of function of *Oshak1* reduced the total uptake of K^+ by 80%, under conditions of K^+-deficiency, and to lesser extent, by 65% under K^+-sufficient supply, the result being an increased plant sensitivity to salt stress. H_2O_2 production in plants increases under conditions of K^+-deficiency (Foreman et al., 2003; Kim et al., 2010; Kurusu et al., 2015; Shin and Schachtman, 2004). Shin and Schachtman (2004) found that the suppression of the NADPH oxidase (*rhd2*) prevented the up-regulation of genes that are normally involved in K^+ uptake under HATS, while the addition of H_2O_2 restored the expression of genes induced by K^+-deficiency in the *rhd2* mutants, and was also sufficient to induce HATS activity under conditions of K^+ sufficiency. Another study showed that RC13, a member of the type III peroxidase family, is involved in the production of ROS, when there is insufficient K^+, and that it is this RCI3-mediated ROS production that regulates the expression of *AtHAK5* (Kim et al., 2010).

Most stresses cause an increase in apoplastic H_2O_2 production, which, in turn, induces Ca^{2+} signature via the H_2O_2-responsive hyperpolarisation-activated Ca^{2+} channels. This Ca^{2+} signal can be amplified by Ca^{2+} activation of the plasma membrane NADPH oxidases, by binding with the EF-hand, resulting in an increase in apolplastic $OH^{\bullet}$ production, which, in turn, causes further Ca^{2+} influx through Ca^{2+} permeable channels (Demidchik et al., 2003; Demidchik et al., 2007).

Several studies have revealed that Respiratory burst oxidase homologs (Rbohs) proteins are involved in different signaling pathways, including root-hair growth, stomatal closure, and plant adaptation to various abiotic stresses (Foreman et al., 2003; Miller et al., 2009; Monshausen et al., 2007; Suzuki et al., 2011; Torres and Dangl, 2005). These proteins encode NADPH oxidases that play an important role in the signaling network of ROS production in plants (Baxter et al., 2014). Rbohs proteins also play a key role in the Ca^{2+}-ROS signaling network, during stress adaptation, via protein phosphorylation and Ca^{2+} (Kimura et al., 2012). Rbohs are basically

activated in two ways: by the Ca^{2+} binding to the EF-hand motif, and by phosphorylation (Ogasawara et al., 2008).

Monshausen et al. (2007) showed that mechanical stimulation triggers pH change, but not the endogenous increase in ROS in the trichoblasts of the Arabidopsis root-hair mutant, which lack the functional *RbohC*. This suggested that the ROS production in the cell wall is the result of mechanical stimulation that up-regulates the *RBOHC* activity, by raising the cytosolic Ca^{2+} concentration.

The potassium content of agricultural soil is estimated at between 1-2% , of which only 2-10% is available for plant uptake since most of the K^+ is bound in the crystal-lattice structure of minerals (Mengel et al., 2001). However, it has been established that, a high cytosolic K^+ concentration of between 100 and 200 mM is a prerequisite to ensure ideal conditions for plant growth and development (Hawkesford et al., 2012). Therefore, the plant's ability to facilitate a high-affinity K^+ uptake is vital, particularly under low K^+ conditions. Furthermore, it has been reported that AtHAK1 mediates high-affinity K^+ uptake and as well as low-affinity Na^+ uptake. On the other hand, saline conditions would both disturb K^+ uptake and reduce K^+ availability for plant uptake, because of strong competition between Na^+ and K^+ on the K^+ binding sites located in the root surface of the plants. Another important consideration is the fact that a plant's ability to withstand salt tolerance is a complex trait involving the responses of a large numbers of genes. Therefore, any modification, even in a single gene, would have a knock-on effect on the other genes responding to the same stimuli. Oxidative stress is a component of salinity stress. In view of this, it would be worth investigating whether a loss of function of the *OsHAK* genes could alter the expression level of *Rbohs* genes. A previous study has shown that lack of *RbohF* function causes salt-induced ROS deficiency in the roots of Arabidopsis, thereby increasing stellar and sap Na^+ concentration. The result is a steep raise in Na^+ content of shoots, and eventually, a sharp increase in plant hypersensitivity to salinity (Jiang et al., 2012).

The aim of the present study is to determine the electrophysiological roles of OsHAK1 and OsHAK5 in rice K^+ uptake, for HATS and LATS ranges of K^+ concentrations, and under salt and oxidative stress conditions. The results provide strong evidence that OsHAK1 and OsHAK5 have dual effects on plant K^+ uptake: loss of function of *Oshak1* and *Oshak5* resulted in a higher K^+ efflux, under both HATS and salt condition; on the other hand, loss of function of *Oshak1* and *Oshak5* down-regulated the expression level of the respiratory burst oxidase homolog OsRbohs proteins, under low K^+ conditions, resulting in lower K^+ efflux compared to the wild type under oxidative stress conditions.

3.2 Materials and Methods

3.2.1 Plant materials

Mature seeds of *Oryza sativa L. Japonica* cv Dongjin wild type (DJ), and its knock-out mutants *Oshak1* and *Oshak5*, and those of *O. sativa L. Japonica* cv Hwayoung wild type (HY), and its knock-out mutant *Oshak5* were obtained from State Key Laboratory of Crop Genetics and Germplasm Enhancement, MOA Key Laboratory of Plant Nutrition and Fertilization in Lower-Middle Reaches of the Yangtze River, College of Resources and Environmental Sciences, Nanjing Agricultural University, Nanjing, China. The seeds of mutants and wild type used in the present study all originated from plants grown under identical conditions.

3.2.2 [1]Detection of transcriptional expression of the *OsHAK1* and *OsHAK5* genes in rice plants

The total RNA was extracted from the root and shoot tissues, and, the reverse transcription RT-PCR, and q RT-PCR for the *OsHAK1* and *OsHAK5* genes was carried out, according to the methods described in the study by Ai et al. (2009); (Tang et al., 2012b). All primers for the semi-quantitative RT-PCR and quantitative real-time PCR are explained in detail in the supporting information section at the end of the thesis.

3.2.3 [2]Whole-plant physiological assessment

To test the hypothesis that *OsHAK5* and *OsHAK1* genes play an important role in plant salt tolerance, rice plants, both wild type and the *Oshak5, Oshak1* mutants were grown in an IRRI solution (Yoshida et al., 1976) containing the following elements: 1.25 mM NH_4NO_3, 0.3 mM KH_2PO_4, 0.35 mM K_2SO_4, 1 mM $CaCl_2$, 1 mM $MgSO_4$ 0.5 mM Na_2SiO_3, 20 mM NaFeEDTA, 20

[1] These data are fully explained in the supporting information section.

[2] Due to limited number of seeds available, all phenotyping experiments for this chapter were conducted by our Chinese collaborators at Nanjing Agricultural University, while our focus in Hobart was on electrophysiological studies

mM H_3BO_3, 9 mM $MnCl_2$, 0.32 mM $CuSO_4$. Ten-day-old seedlings of *Oshak5* and its wild type were grown in IRRI solution containing 1 mM K for 3 weeks, then transferred to a solution containing additionally 100 mM NaCl and allowed to grow for 8 d. While, the rice seedling of *Oshak1* mutant and its wild type were grown in the IRRI solution containing 1 mM K for 4 weeks and then transferred into a solution in which K_2SO_4 and/or KH_2PO_4 were replaced by Na_2SO_4 and/or NaH_2PO_4, respectively. For long-term NaCl treatment, rice plants were gradually exposed to NaCl over 2 d to reach a final concentration of 50 mM in the first time. The solution was replaced every 2 d. All the plants were grown in a greenhouse with a 16-h-light (30°C)/ 8-h-dark (22°C) photoperiod, and the relative humidity was controlled at 60% to 70%.

3.2.4 Microelectrodes preparation

The microelectrodes were prepared as described in the first experimental chapter. The electrode tip was front-filled with the corresponding LIX which listed in the following table (Table 3.1)

Table 3. 1 Ionophores (LIX) and the back-filling solutions which were used to prepare the microelectrode of the selected ion

Ion	Ionophore (LIX)	Back-filling solution (mM)
K^+	Valinomycin	500 KCl
Ca^{2+}	(-)-(R,R)-N,N′-(Bis(11-ethoxycarbonyl)undecyl)-N,N′-4,5-tetramethyl-3,6- dioxaoctanediamide	500 $CaCl_2$
H^+	4-Nonadecylpyridine	15 NaCl+ 40 KH_2PO_4, (pH 6)

To calibrate the microelectrodes, an appropriate set of three standard solutions, (Table 3.2) covering the expected range of targeted ion was prepared. The procedure of calibration is described in the first experimental chapter.

Table 3. 2 Measured ions and their standard calibration solutions

Ion	Standard calibration solutions
Ca^{2+}	100, 200, 400 µM $CaCl_2$
K^+	250, 500, 1000 µM KCl
H^+	Buffers with pH 5.30, 6.67, 7.65

3.2.4.1 Experimental protocols

Root preparation was carried out as described in the first experimental chapter. The growth media used for seeds germination contained two concentrations of potassium (50 µM, and 5 mM KCl). The BSM bathing medium consisted of 200 µM NaCl, 100 µM $CaCl_2$, and 200 µM KCl. The pH level of the BSM solution was ~5.4. For conditioning, the roots were left in the bathing solution for approximately 30-60 min.

The measurement was carried out as described in the first experimental chapter. The treatments were 40 mM NaCl and 10 mM H_2O_2. The ion flux was measured in both the root mature and the elongation zones.

3.2.4.2 Measurement of membrane potential (*Em*)

A blank microelectrode, with a tip diameter ≈0.5 µm, was filled with 0.5 M KCl, and connected to the MIFE system by means of the Ag/AgCl wire. The microelectrode tip was manually inserted into the epidermal cells of the mature zone of the rice root. The measurements were taken on the root, firstly, under the normal condition (a standard BSM, as previously described), and subsequently, when the root was exposed to 40 mM NaCl. The measurements were taken at time intervals of between 1 and 30 min. Five replicates (roots) were used for each treatment, with 3-5 cell measurements being taken of each individual replicate (Cuin and Shabala, 2005).

3.3 Results

3.3.1 Loss of function of *Oshak5* and *Oshak1* resulted in growth impairment in rice plants under salt stress conditions.

A whole-plant physiological assessment showed that the *Oshak5* mutants had a significantly ($P \leq 0.01$) lower total dry weight by 33-37% compared to the wild types under saline condition. This resulted from a significant reduction in the root and shoot dry weight of both mutants (Table 3.3).

Table 3. 3 Dry weight (g plant^{-1}) of wild types (DJ) and (HY) and *Oshak5* mutant plants under saline conditions. Ten-day-old seedling were grown in IRRI solution containing 1 mM K for 3 weeks, then transferred to a solution containing 100 mM NaCl where they were grown for a further 8 days. Data are the mean ± SE (n=5). Different letters are used to indicate the significant differences between the lines ($P \leq 0.05$, one-way ANOVA).

Lines	Root	Shoot	Total
	DW (g plant^{-1})		
WT(DJ)	0.23±0.038a	1.03±0.069a	1.26±0.09a
Oshak5	0.15±0.036b	0.65±0.068b	0.80±0.08b
WT(HY)	0.19±0.035a	0.91±0.070a	1.10±0.09a
Oshak5	0.14±0.029b	0.60±0.068b	0.74±0.07b

Table 3. 4 Dry weight (g plant^{-1}) and the total K^+ content of wild types (DJ) and (HY) and *Oshak5* mutant plants, under low K^+ condition. Ten-day-old seedling were grown in IRRI solution containing 1 mM K for 2 weeks, then transferred to a solution containing 0.3 mM, where they were grown for a further 2 weeks. Data are mean ± SE (n=5). Different letters are used to indicate the significant differences between the lines ($P \leq 0.05$, one-way ANOVA).

	Total dry weight	Total K^+
Lines	g plant^{-1}	(mg g^{-1} DW)
WT(DJ)	1.07±0.04a	25.0±1.9a
Oshak5 (DJ)	0.58±0.02b	14.3±2.1b
WT(HY)	1.31±0.04a	35.7±2.8a
Oshak5 (HY)	0.71±0.03b	17.9±1.8b

The study also compared between the *Oshak5* mutant lines and their respective wild types grown in a nutrient solution containing 0.3 mM K. the result showed that the loss of function of *Oshak5* impaired the growth of the both mutant lines as compared to their respective wild types. In addition to that, due to a significant reduction in biomass accumulation, the total K^+ content was only about 56% to 60% in the both mutant lines as compared to their respective wild types, under low K condition (Table 3.4).

The physiological assessment of the role of OsHAK1 in plant salt tolerance showed that loss of function of *Oshak1* caused a significant ($P \leq 0.05$) reduction in the total dry weight of the mutant plants compared to the wild type, regardless of the external K^+ concentration (Table 3.4). Interestingly, under conditions of K^+ deficiency, this reduction in dry weight was more pronounced in mutant plants than in the wild type by almost 60%, while under normal K^+ conditions, it was only 45% lower than in the wild type (Table 3.4). Thus, loss of function of the *Oshak5* and *Oshak1* genes resulted in a greater increase in the sensitivity of the mutant plants to salt stress than in that of the wild type. This sensitivity was also much stronger under K^+-deficient conditions.

Table 3. 5 Dry weight (g plant^{-1}) of wild type (DJ) and *Oshak1* mutant plants under saline conditions. Two-week-old seedling were grown in IRRI solution containing 1 mM K for 4 weeks, then transferred to a solution containing either 1 mM K and 50 mM NaCl or 0.1 mM K and 50 mM NaCl, where they were grown for a further 4 weeks. Data are the mean ± SE (n=18). Different letters are used to indicate the significant differences between the lines ($P \leq 0.05$, one-way ANOVA).

	Lines	(mM) 0.1K+50 Na	1K+50 Na
		DW (g plant^{-1})	
Root	WT(DJ)	0.53±0.05b	0.70±0.02a
	Oshak1	0.24±0.02d	0.40±0.04c
Shoot	WT(DJ)	3.29±0.28b	4.77±0.28a
	Oshak1	1.36±0.42d	2.61±0.15c
Total	WT(DJ)	3.82±0.25b	5.47±0.02a
	Oshak1	1.60±0.31d	3.01±0.11c

3.3.2 The salt sensitivity of *Oshak* mutants is linked to low shoot K^+:Na^+ ratio

The results of the root and shoot K^+ and Na^+ content of *Oshak5* mutants and their wild type clearly explain the growth reduction of *Oshak5* mutants compared to the wild types, under salt stress conditions. The *Oshak5* mutants had a similar root Na^+ content, but a 30-38% higher shoot Na^+ content compared to wild types (Table 3.5). However, in terms of root and shoot K^+ content, no significant difference was noted between the lines. Therefore, under salt conditions, the K^+:Na^+ ratio was lower by 25-30% in the *Oshak5* mutants compared to the wild types (Table 3.5).

Table 3. 6 K^+, Na^+ content and K^+:Na^+ ratio of wild types (DJ) and (HY) and *Oshak5* mutant plants under saline conditions. Ten-day-old seedling were grown in IRRI solution containing 1 mM K for 3 weeks, then transferred to a solution containing 100 mM NaCl where they were grown for a further 8 days. Data are the mean ± SE (n=5). Different letters are used to indicate the significant differences between the lines ($P \leq 0.05$, one-way ANOVA).

	K^+ (mg g^{-1} DW)		Na^+ (mg g^{-1} DW)		K^+/Na^+ ratio	
Lines	Root	Shoot	Root	Shoot	Root	Shoot
WT(DJ)	22.9±1.0a	30.3±0.9a	16.8±0.9a	13.7±1.0b	1.36±0.28a	2.21±0.23a
Oshak5	23.8±1.1a	29.4±1.3a	17.9±0.4a	18.9±1.6a	1.33±0.11a	1.55±0.09b
WT(HY)	21.3±0.5a	28.8±0.7a	16.8±1.0a	14.7±0.9b	1.26±0.20a	1.95±0.12a
Oshak5	21.2±0.5a	28.7±0.8a	17.3±0.7a	19.2±2.1a	1.22±0.15a	1.47±0.08b

In the case of the *Oshak1* mutant, exposure to 50 mM NaCl decreased both root and shoot K^+ content by 61%, and 50%, respectively (Table 3.6). At the same time, root and shoot Na^+ content increased by 29%, and 73%, respectively, in the *Oshak1* mutant plants compared to the wild type under normal K^+-supply conditions (Table 3.6). Reducing the external K^+ concentration to 0.1 mM resulted in a further decrease in root and shoot K^+ content, by up to 64%, and 58%, respectively. This was accompanied by a further increase in root and shoot Na^+ content by 36%, and 85%, respectively compared to the wild type (Table 3.6). Therefore, the K^+:Na^+ ratio was significantly ($P \leq 0.01$) lower by 77% in the *Oshak1* mutant under K^+-deficient conditions, and by approximately 70% under normal K^+-supply conditions compared to the wild type.

Table 3. 7 K^+, Na^+ content and K^+/Na^+ ratio of wild type (DJ) and *Oshak1* mutant plants under saline conditions. Two-week-old seedling were grown in IRRI solution containing 1 mM K for 4 weeks, then transferred to a solution containing either 1 mM K and 50 mM NaCl or 0.1 mM K and 50 mM NaCl, where they were grown for a further 4 weeks. Data are the mean ± SE (n=5). Different letters are used to indicate the significant differences between the lines ($P \leq 0.05$, one-way ANOVA).

		K^+ (μmol g^{-1} DW)		Na^+ (μmol g^{-1} DW)		K^+/Na^+ ratio	
	Lines	0.1K+50Na	1K+50Na	0.1K+50Na	1K+50Na	0.1K+50Na	1K+50Na
Root	WT(DJ)	132±11b	276±25a	475±21b	398±16c	0.28±0.02b	0.69±0.05a
	Oshak1	48±03c	107±10b	645±47a	513±38b	0.07±0.03d	0.20±0.01c
Shoot	WT(DJ)	343±34b	615±48a	701±47c	541±35d	0.48±0.08b	1.14±0.12a
	Oshak1	143±15c	305±19b	1294±85a	936±71b	0.11±0.01d	0.33±0.04c

3.3.3 Loss of function of *Oshak1* and *Oshak5* resulted in low K^+ uptake under both salt stress and high-affinity potassium conditions

OsHAK1 and *OsHAK5* are both members of the *KUP/HAK/KT* family of potassium transporters, and according to Gupta et al. (2008) are involved in high-affinity K^+ uptake. Therefore, to test this, the present study used the MIFE technique to study the role of OsHAK1 and OsHAK5 in K^+ acquisition, under both salt stress and low and high-affinity potassium conditions in WT and knock-out mutant lines.

Under high-affinity potassium conditions (50 µM K^+), salt treatment significantly altered the ions fluxes, measured both in the elongation and mature zones of the rice plants.

NaCl induced a significantly higher K^+ efflux from the elongation zone of the *Oshak1* and *Oshak5* mutants compared to their wild types (Fig 3.1). However, it reduced the initial K^+ efflux in the wild types, by between 15 and 40 $nmolm^{-2}s^{-1}$ in (DJ) and (HY) respectively, compared to those under the non-treated condition (Fig 3.1a.c). In contrast, loss of function of *Oshak1* and *Oshak5* resulted in a significant initial K^+ efflux of between -50 and -160 $nmolm^{-2}s^{-1}$, which was

3-4 times higher than in the wild types, followed by a gradual recovery throughout the remainder of the measurement duration (Fig 3.1a.c).

Under the low-affinity potassium condition (5 mM K), the *Oshak1* and *Oshak5* mutants and their wild types exhibited contrasting responses, in the elongation zone in response to salt treatment compared to the high-affinity potassium condition (Fig 3.1b.d). Salt treatment caused a K^+ efflux in the wild types, which ranged between -36 to -110 $nmolm^{-2}s^{-1}$, while the *Oshak1* and *Oshak5* mutants showed a lower initial K^+ efflux of approximately -27 $nmolm^{-2}s^{-1}$ compared to the K^+ efflux before salt treatment. No significant difference was detected between the tested lines, under the HATS condition (Fig 3.1b.d). The starting fluxes of different ions may vary depending on the health status of the seedling, the part of the root where the measurement was taken, the quality of the microelectrode which have been used to carry out the measurement, and the temperature of the bathing media and the standard solutions that have been used for calibration.

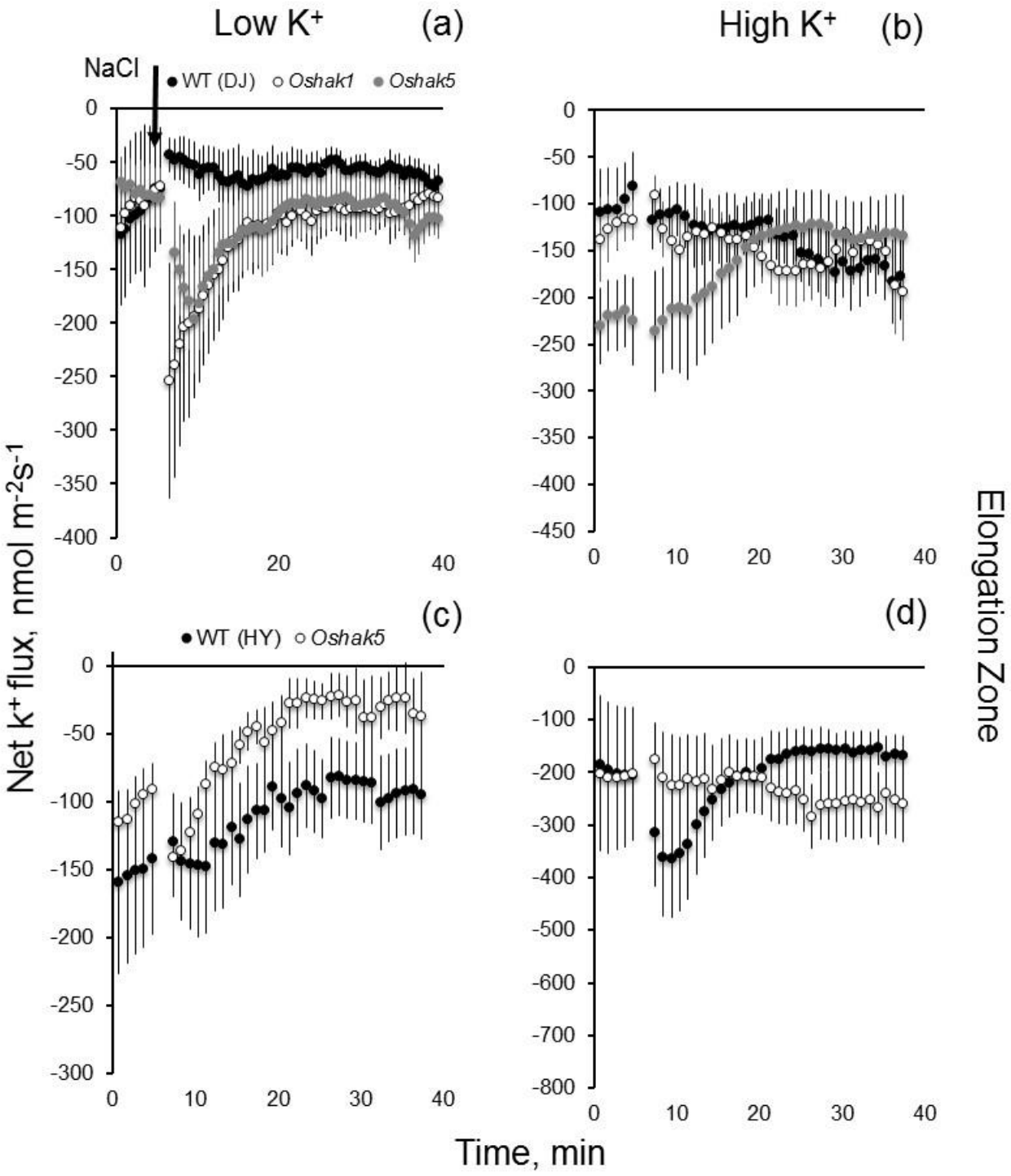

Figure 3. 1 Salt-induced K^+ flux in the elongation zone of wild type (DJ), *Oshak1* and *Oshak5* mutants (a, b), and of wild type (HY) and the *Oshak5* mutant (c, d) under a high-affinity- potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day -old seedlings of all lines were treated with 40 mM NaCl for 30 minutes. Data are the mean ± SE (n=5).

The response to salinity in the mature zone of the wild types was the opposite of that in the elongation zone (Fig 3.2). Here, NaCl induced an initial K^+ efflux, of about -15 $nmolm^{-2}s^{-1}$ compared to the non-treated condition (Fig 3.2a.c) while the *Oshak1* and *Oshak5* mutants of the wild type (DJ) showed a significantly greater K^+ efflux, by one-fold compared to their wild type (Fig 3.2a). There was no significant difference in K^+ efflux between the wild type (HY) and its mutant under a high-affinity potassium range (Fig 3.2c).

In the mature zone, the addition of 40 mM NaCl caused an increase in K^+ efflux that was greater in all lines, than it was pre-treatment. However, upon salt exposure to salt and under low-affinity potassium range, no significant difference was recorded between the wild type (DJ) and its *Oshak1* and *Oshak5* mutants, while K^+ efflux was higher in the *Oshak5* mutant than in its wild type (HY), (Fig 3.2.d).

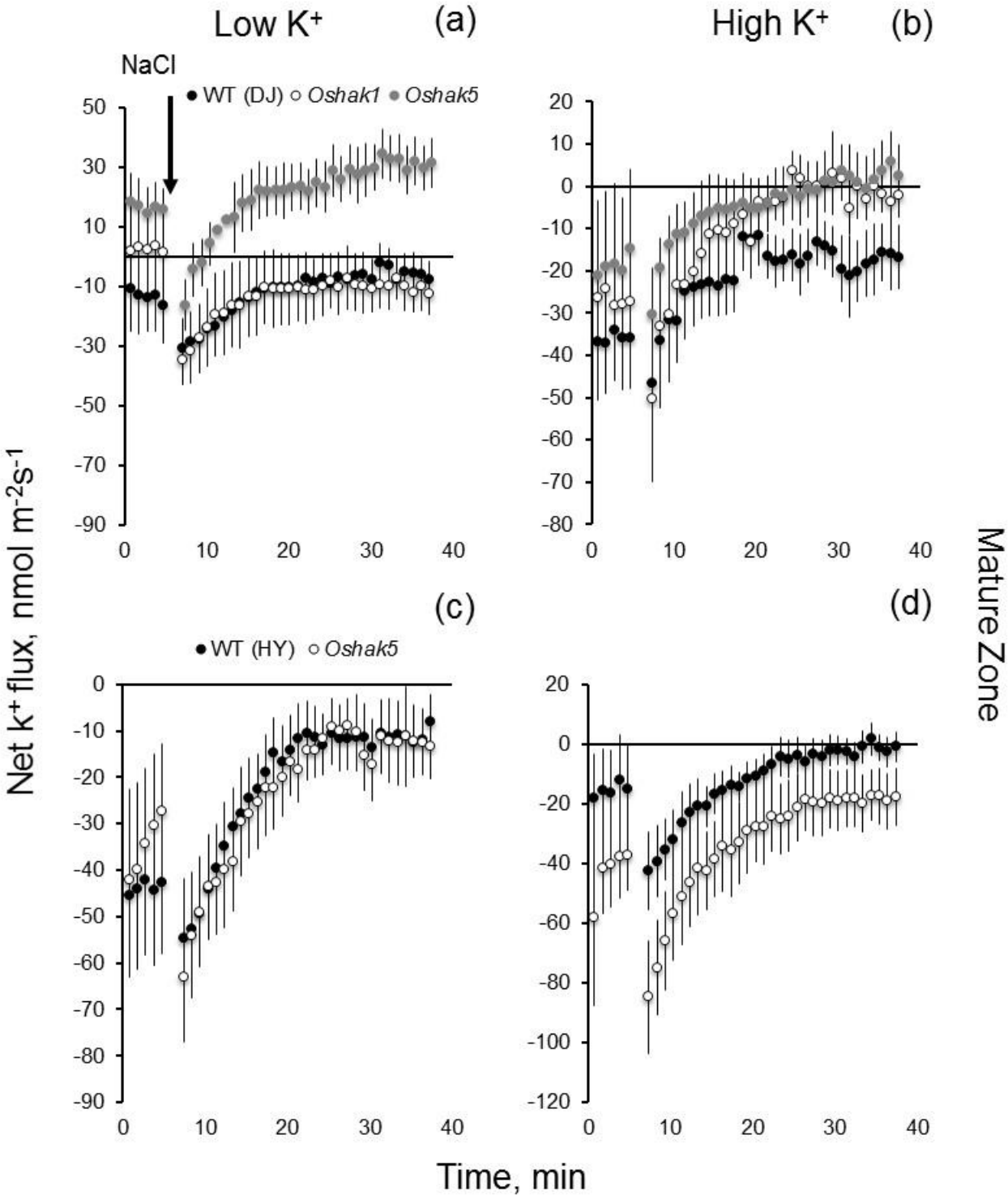

Figure 3. 2 Salt-induced K^+ flux in the mature zone of wild type (DJ), *Oshak1* and *Oshak5* mutants, and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 40 mM NaCl for 30 minutes. Data are the mean ± SE (n=5).

It has been reported that K^+ flux responses differ, depending on the functionality of the various root zones (Chen et al., 2005). In accordance with this, the K^+ effluxes from the mature zone of all lines at between -10 and -60 $nmolm^{-2}s^{-1}$, were 4-10 times lower than those from the elongation zone, which were between -100 and -250 $nmolm^{-2}s^{-1}$.

3.3.4 Loss of function of *Oshak1* and *Oshak5* resulted in higher H^+ efflux both under salt stress and high-affinity potassium conditions

Salinity also caused a reduction in H^+ flux, in the elongation and mature zones of all lines, both under low and high-affinity potassium conditions. Additionally, each genotype responded differently to salt stress. In the elongation zone and under high-affinity potassium conditions, neither the wild type (DJ) nor the *Oshak1* mutant responded directly to salt treatment. Meanwhile, NaCl caused a gradual reduction, over time, in the H^+ flux, in both lines. However, the effect was greater on the *Oshak1* mutant compared to its wild type. Meanwhile, the *Oshak5* mutant's response to the salt treatment was immediate: the H^+ influx changed to efflux, resulting in a higher H^+ efflux compared to the wild type (DJ) (Fig 3.3a). In response to salt treatment, both the wild type (HY) and the *Oshak5* mutant experienced an immediate reduction in their H^+ influx, but with no significant difference between the two responses (Fig 3.3c).

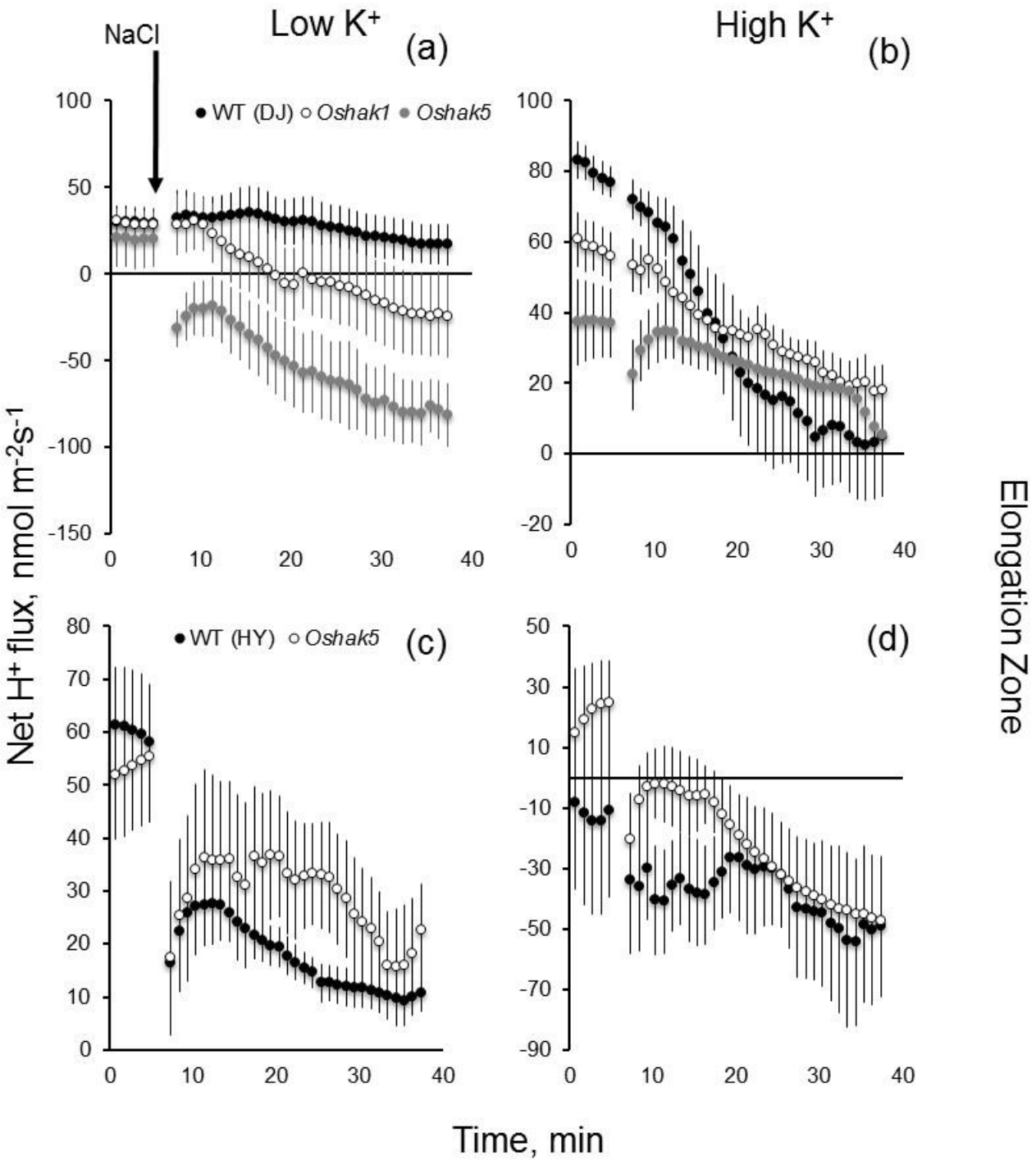

Figure. 3 Salt-induced H^+ efflux in the elongation zone of wild type (DJ) and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 40 mM NaCl for 30 minutes. Data are the mean ± SE (n=5).

Surprisingly, under high-affinity potassium conditions, the H^+ flux was negative in the mature zone of the non-treated roots of all lines, except the wild type (HY) (Fig 3.4). In the mature

zone of the wild types, the response of the H^+ flux was similar to that in the elongation zone. The *Oshak1* and *Oshak5* mutants immediately showed a higher H^+ efflux compared to those under the non-treated condition, and a larger H^+ efflux as compared to wild type (DJ) (Fig 3.4a).

Salinity induced a rapid decrease in H^+ influx in the wild type (DJ) from 82 $nmolm^{-2}s^{-1}$ to 5 $nmolm^{-2}s^{-1}$, while the H^+ influx reduction was to lesser range in the elongation zone of *Oshak1* and *Oshak5* mutants, under the low-affinity potassium condition (Fig 3.4b). However, the reduction in H^+ influx was more significant in the wild type (DJ) than in the *Oshak1* and *Oshak5* mutants. A similar reduction occurred in the wild type (HY) and its *Oshak5* mutant, but in this case, there was no significant difference between them (Fig 3.4c).

In its mature zone, the *Oshak5* mutant showed evidence of a H^+ influx, while its wild type (HY) showed a high H^+ efflux, under the LATS and salt conditions. This resulted in a significant difference between both lines (Fig 3.4d). Wild type (DJ), *Oshak1*, and *Oshak5* all responded non-significantly to NaCl treatment, with the H^+ influx shifting to efflux (Fig 3.4b).

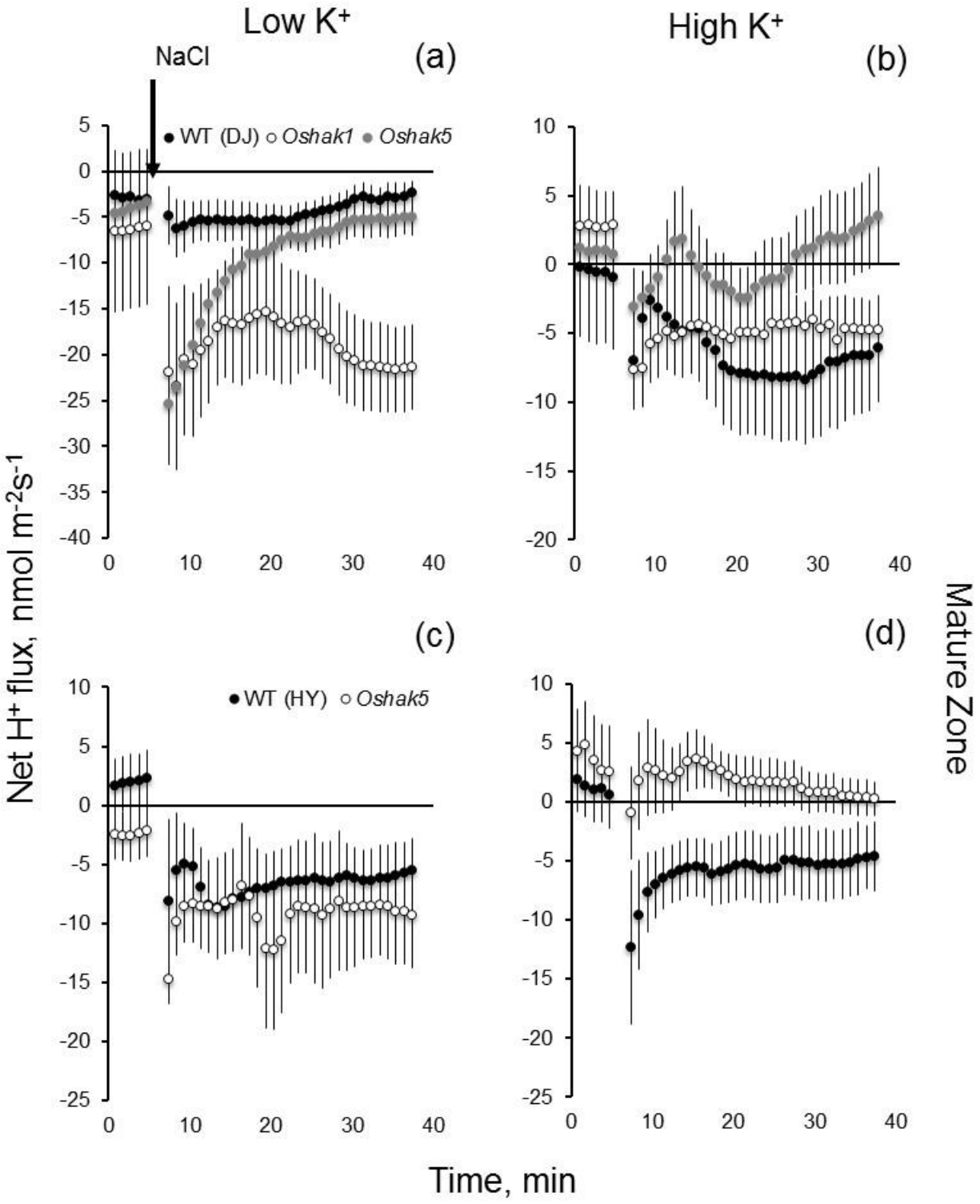

Figure 3. 4 Salt-induced H^+ efflux in the mature zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 40 mM NaCl for 30 minutes. Data are the mean ± SE (n=5).

3.3.5 The responses of ion fluxes to oxidative stress

H_2O_2-induced K^+ efflux showed different genotypic and tissue specificities. Under the control condition, there were no significant differences, between the lines, in terms either of their K^+ efflux in the elongation and mature zones, or their low or high affinity potassium conditions, with the exception of the *Oshak5*, which had a higher K^+ influx than its wild type (HY) in the mature zone (Fig 3.6c).

In the elongation zone, and upon exposure to 10 mM H_2O_2, a dramatic rise in K^+ efflux occurred, under the high-affinity potassium condition. It reached a peak of -624 $nmolm^{-2}s^{-1}$ after 25 min of treatment, and then began to decrease, over the time in the wild type (DJ). A corresponding gradual increase in K^+ efflux was recorded in the *Oshak1* and *Oshak5* mutants, where a peak of -350 $nmolm^{-2}s^{-1}$ was reached after 32 mins of treatment (Fig 3.5a). This result suggests their lower sensitivity to oxidative stress. On the contrary, no significant difference in H_2O_2-induced K^+ efflux was found between the wild type (DJ) and its *Oshak1* and *Oshak5* mutants, under the low-affinity potassium condition (Fig 3.5b). Similarly, wild type (HY) was more sensitive to oxidative stress, and its K^+ efflux was significantly ($P \leq 0.05$) higher compared to that of the *Oshak5* mutants, under both potassium-affinity systems (Fig 3.5c.d).

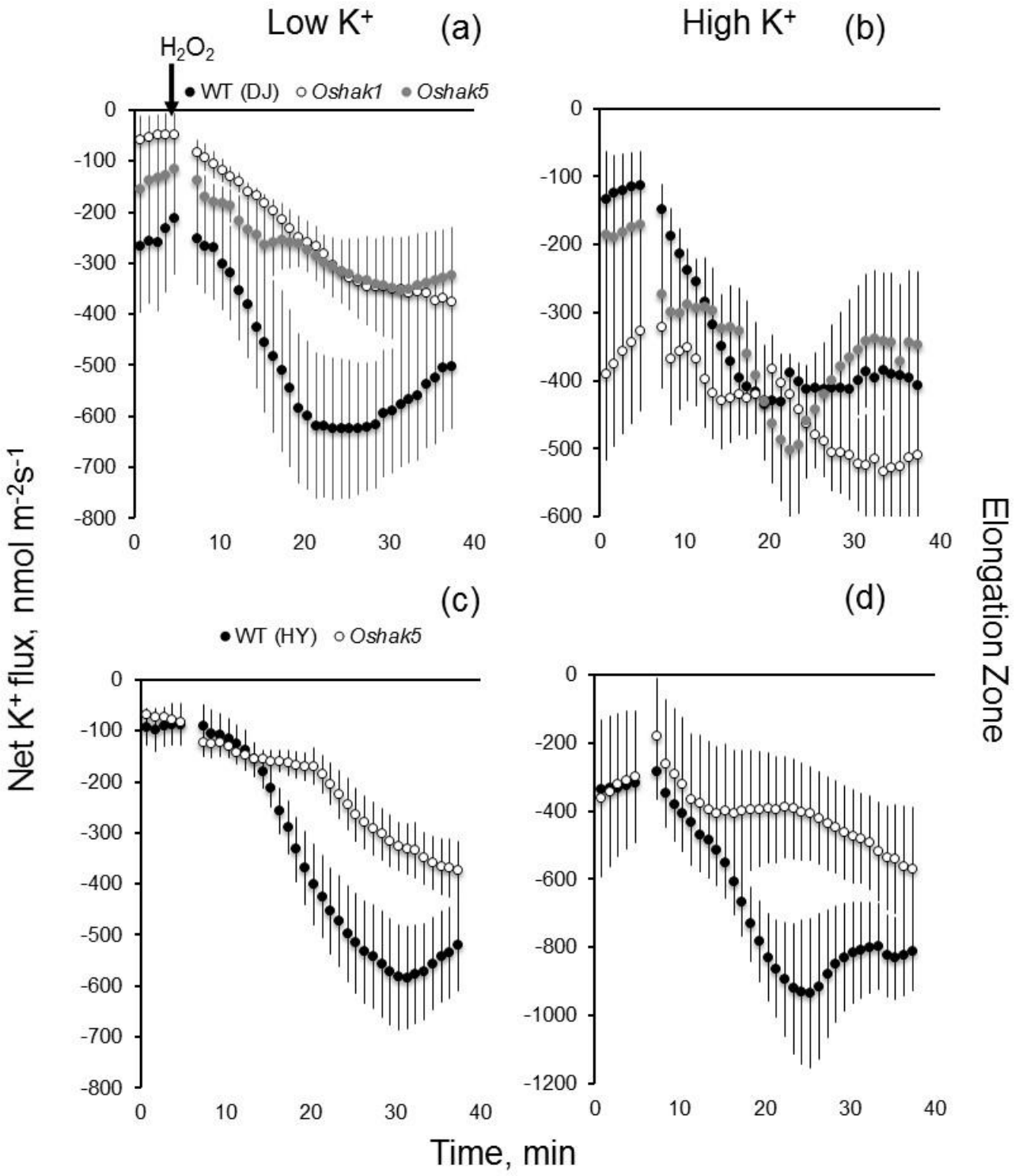

Figure 3. 5 H_2O_2-induced K^+ efflux in the elongation zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a, b), and of wild type (HY) and *Oshak5* mutant (c, d) under a high- affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Data are the mean ± SE (n=5).

In the mature zone, K^+ efflux in the *Oshak1* mutant was observed to be similar to that in the wild type (DJ) under the high-affinity condition. However, 24 minutes after H_2O_2 was introduced, the K^+ efflux began to slowly decrease over the time (Fig 3.6a). Interestingly, it was the *Oshak5* mutant that showed sensitivity to oxidative stress that was significantly higher than that of both of its wild types, with K^+ efflux occurring immediately, reaching a peak of -140 and -90 $nmolm^{-2}s^{-1}$ and then gradually decreasing over the time (Fig 3.6a.c).

Under low-affinity conditions, while no significant difference was noted in K^+ efflux between the wild type (DJ) and its *Oshak1* and *Oshak5* mutants (Fig 3.6b), the *Oshak5* mutant did show evidence of a lower K^+ efflux than the wild type (HY) (Fig 3.6d) which was the opposite trend to the one displayed under the high-affinity system.

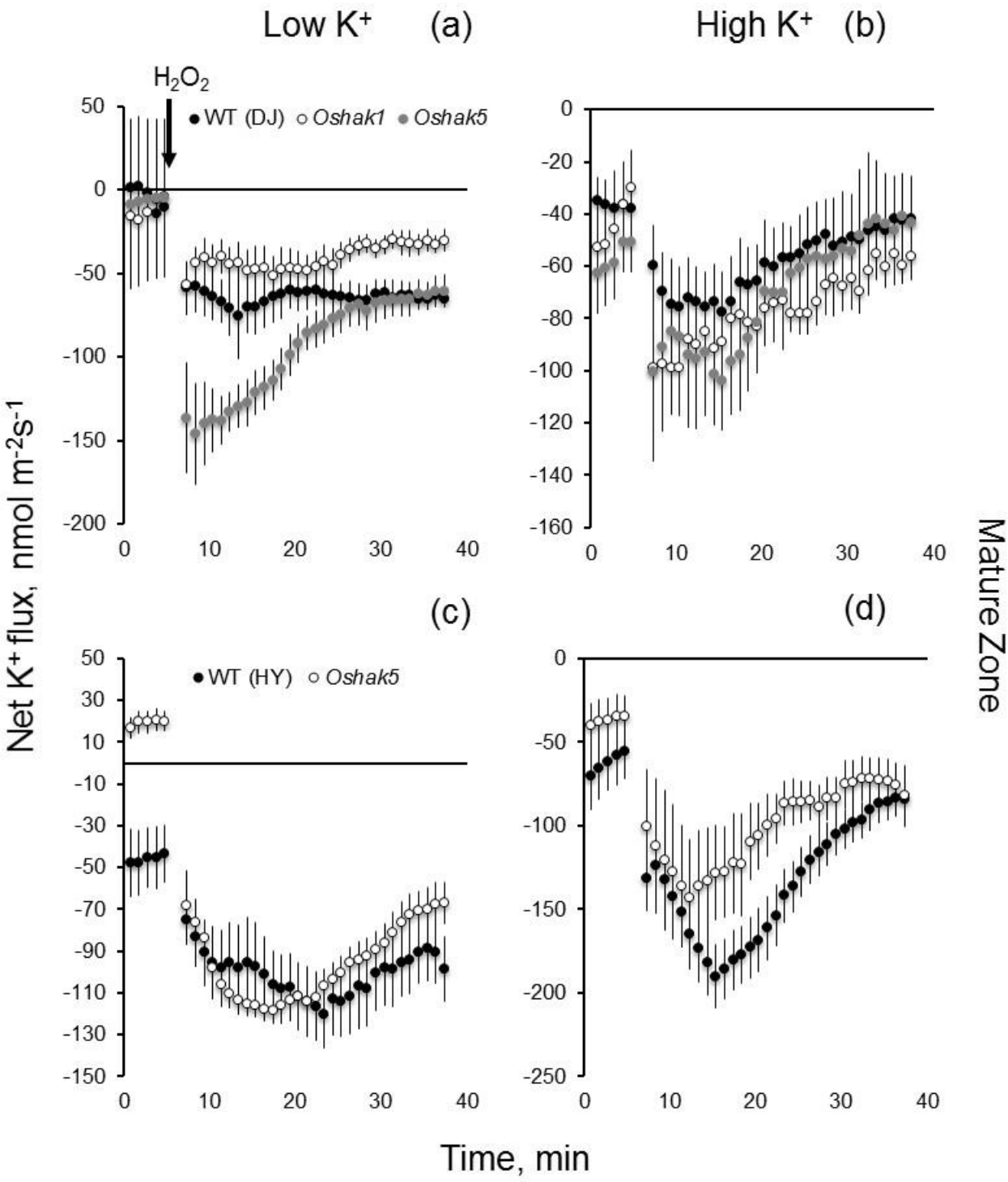

Figure 3. 6 H_2O_2-induced K^+ efflux in the mature zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Mean value ± SE (n=5).

H_2O_2 induced H^+ influx, both in the mature and elongation zones of all lines, under all conditions. However, the response of H^+ flux to oxidative stress was both genotype and tissue-specific.

To compare the lines, 10 mM H_2O_2 was added to the roots of both wild types (DJ and HY) and their mutants (*Oshak1* and *Oshak5*), under the high-affinity potassium condition. This caused a significant ($P \leq 0.05$) increase in H^+ influx in the elongation zone of the wild type (DJ) compared to its mutants (Fig 3.7a). However, no such difference was recorded between the wild type (HY) and its mutant, *Oshak5* (Fig 3.7c).

In contrast, under the low-affinity potassium condition, there was no significant difference in H^+ influx between the lines with the exception of the *Oshak5* mutant, which had a higher H^+ influx than its wild type (HY) in both zones (Fig 3.7d, 8d).

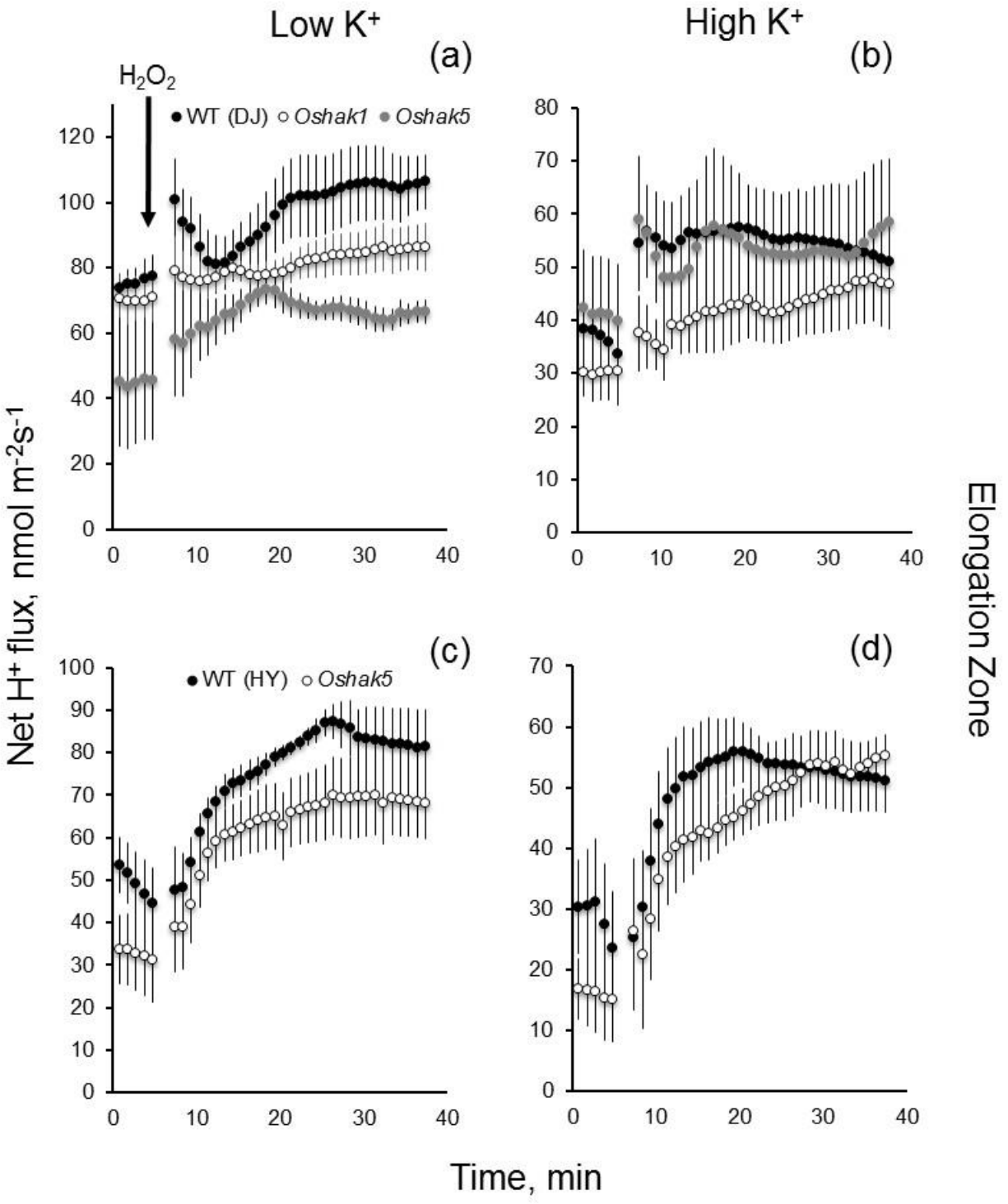

Figure 3. 7 H_2O_2-induced H^+ influx in the elongation zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Mean value ± SE (n=5).

In contrast, H^+ influx in the mature zone of both mutants, *Oshak1* and *Oshak5*, was significantly higher than in that of the wild type (DJ) (Fig 3.8a), a finding that also applied to the *Oshak5* mutant and its wild type (HY) (Fig 3.8c).

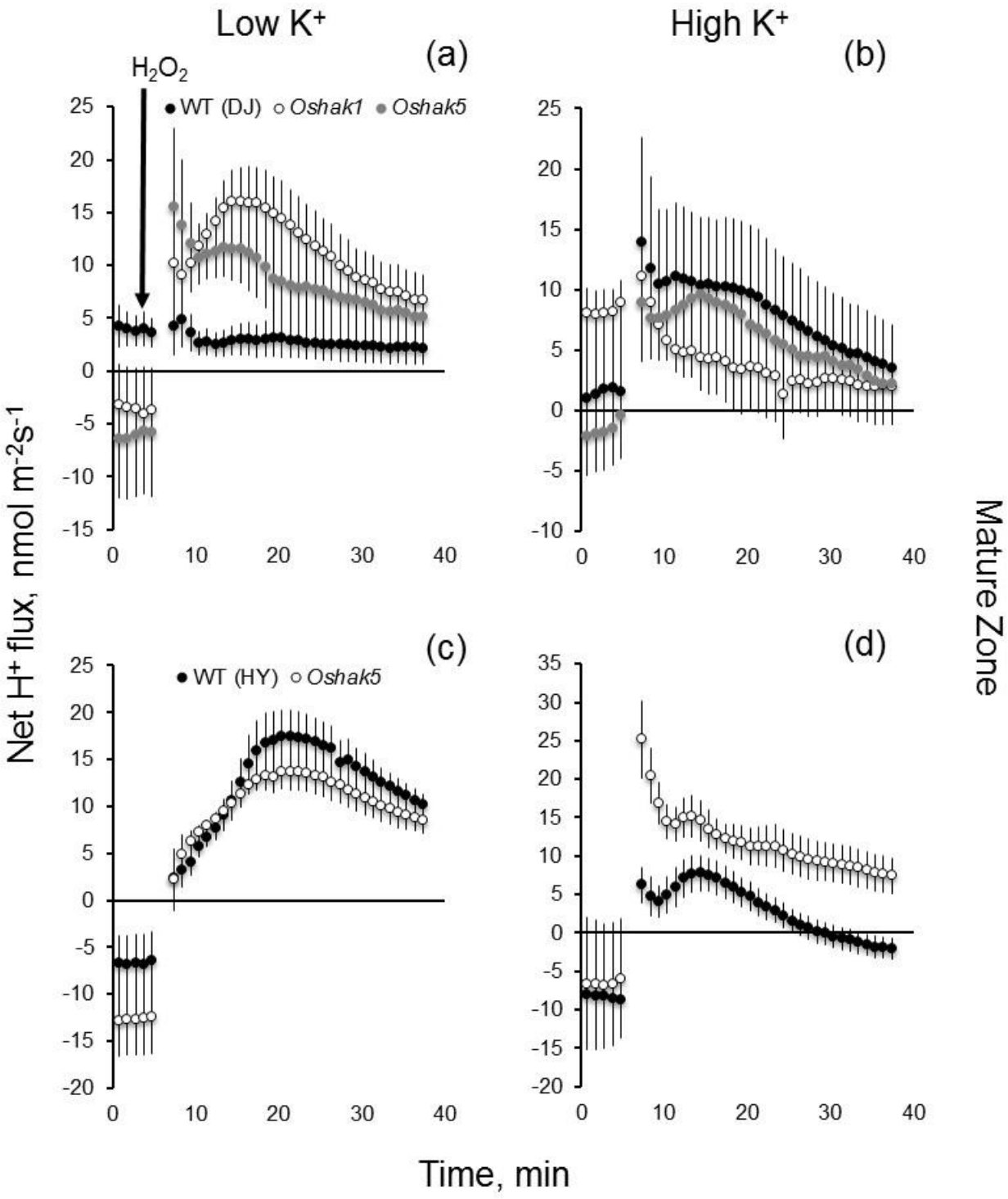

Figure 3. 8 H_2O_2-induced H^+ influx in the mature zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Data are the mean ± SE (n=5).

When the two zones were compared, it was noted that, H_2O_2 induced a gradual H^+ influx which continued for the duration of the experiment in the elongation zone, while in the mature zone this influx was gradual, reaching a peak, which differed depending on the line and then decreasing until the end of the measurement duration (Fig 3.7, 3.8).

Upon exposure to oxidative stress, Ca^{2+} flux displayed a similar trend to that of H^+ flux, in terms of the differences between lines, zones and potassium-uptake systems. Under the high-affinity potassium range, Ca^{2+} influx was higher in the elongation zone of the wild types than in that of the *Oshak1* and *Oshak5* mutants (Fig 3.9a.c), and lower in mature zone, under the same conditions (Fig 3.10a.c). Under the low-affinity potassium condition, no significant difference was observed in terms of Ca^{2+} influx in the elongation zone of all lines (Fig 3.9b.d).

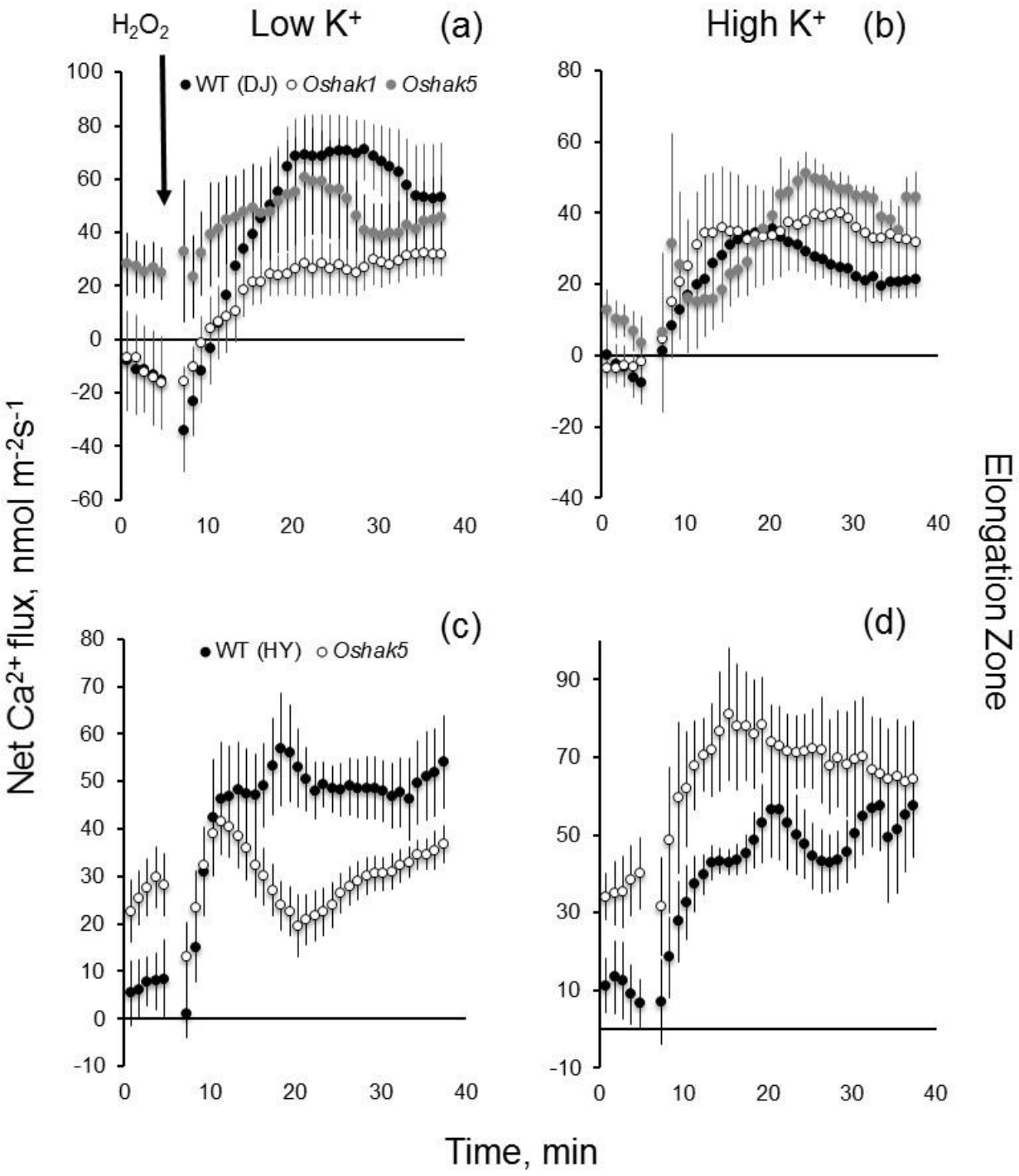

Figure 3. 9 H_2O_2-induced Ca^{2+} influx in the elongation zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a, b), and of wild type (HY) and *Oshak5* mutant (c, d) under a high-affinity potassium system (a, c), and a low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Data are the mean ± SE (n=5).

However, in the mature zone of both *Oshak1* (Fig 3.10b) and *Oshak5* (Fig 3.10d) mutants Ca^{2+} influx was significantly higher than in that of both wild types, (DJ) and (HY) under high affinity K^+ uptake range.

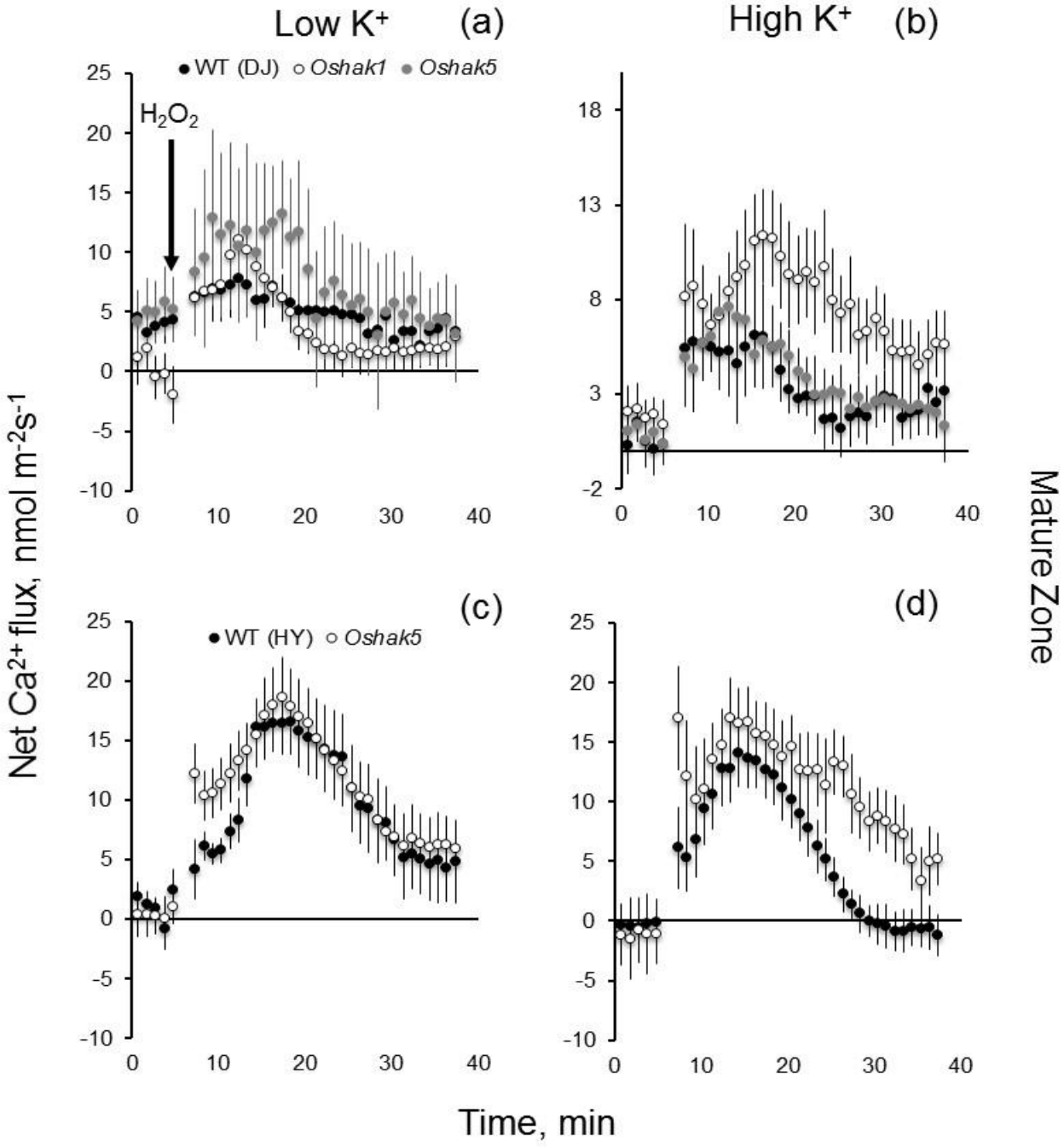

Figure 3. 10 H_2O_2-induced Ca^{2+} influx in the mature zone of wild type (DJ), and *Oshak1* and *Oshak5* mutants (a), and of wild type (HY) and *Oshak5* mutant under a high-affinity potassium system (a, c), and low-affinity potassium system (b, d). 5-6-day-old seedlings of all lines were treated with 10 mM H_2O_2 for 30 minutes. Data are the mean ± SE (n=5).

3.3.6 [3]Expression of *OsRboh* genes under low K^+ conditions

It has been reported that K^+ deficiency causes an increase in the NADPH oxidase that is encoded by *Rbohs* genes in the root cells (Cakmak, 2005). This process results in the generation of ROS, which, in turn, directly activates the GORK and NSCC channels, thereby causing further increase in K^+ efflux. In view of these findings, the present study examined the expression level of *OsRbohs* genes in the roots of the wild type (DJ), and the *Oshak1*, and *Oshak5* mutants, and in wild type (HY) and its *Oshak5* mutant, under low K^+ conditions (0.3 mM) (Fig 3.11). The results showed that the expression levels of *OsRbohA, B, C,* and *G* genes were 7.3, and 9.3 times higher in the wild type (DJ) than in the mutants, *Oshak1*, and *Oshak5*, respectively, while the expression levels of *OsRbohA*, *B*, and *C* were between 1.3 and 1.5 times higher in the wild type than in the *Oshak5* (HY) mutant. Interestingly, the expression of *OsRbohG* is significantly higher in the *Oshak5* (HY) mutant than in the wild type.

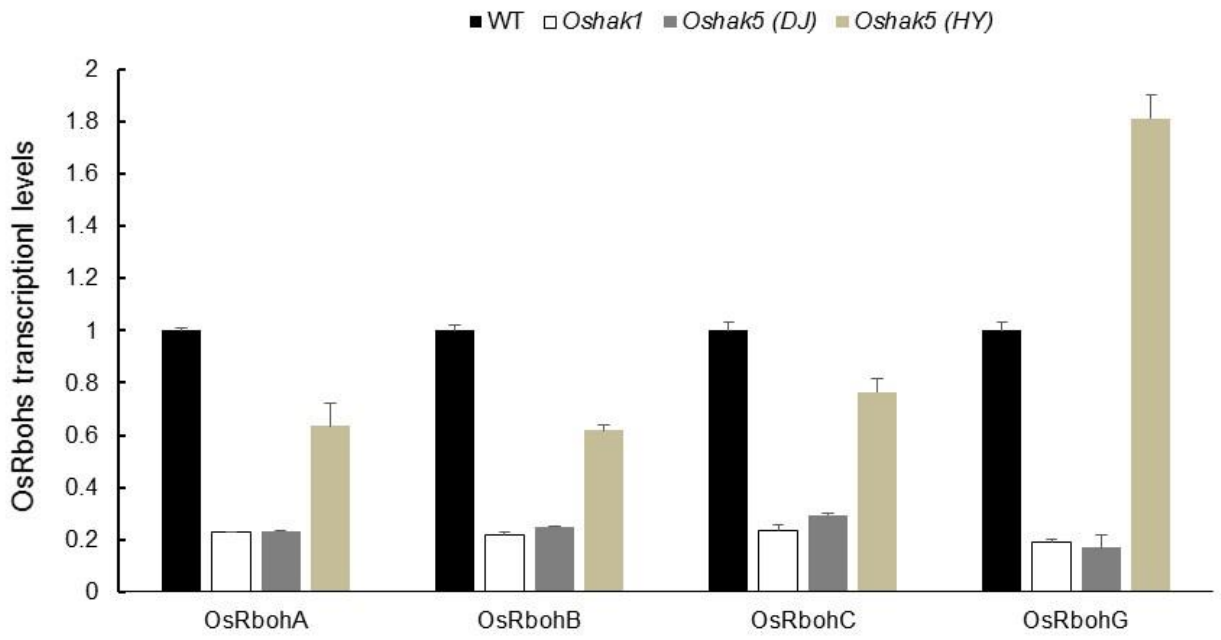

Figure 3. 11 The expression level of *OsRbohs* (A, B, C, G) genes, in the root of wild type (DJ), and *Oshak1*, and *Oshak5* mutants under 1 mM K conditions.

[3] The expression level of *OsRbohs* genes was examined by our collaborators in China

3.3.7 Membrane potential

Under the control condition, the membrane potential (MP) ranged between -110 and -115 mV, in the wild type (DJ) (Fig 3.12), and the *Oshak1*, and *Oshak5* mutants, while it was approximately -90 mV, in both the wild type (HY) and *Oshak5* mutant (Fig 3.13). Adding 40 mM NaCl to the bath medium resulted in a substantial membrane depolarisation after 3 mins. The MP decreased by 25-30% in the wild type (DJ), and *Oshak1*, and *Oshak5* mutants (Fig 3.12), and between 29 and 35 % in the wild type (HY) and the *Oshak5* mutant (Fig 3.13). There followed a further gradual decrease, which finally stabilised towards the end of the measurement phase. No clear difference was observed between the lines, in response to the salinity treatment.

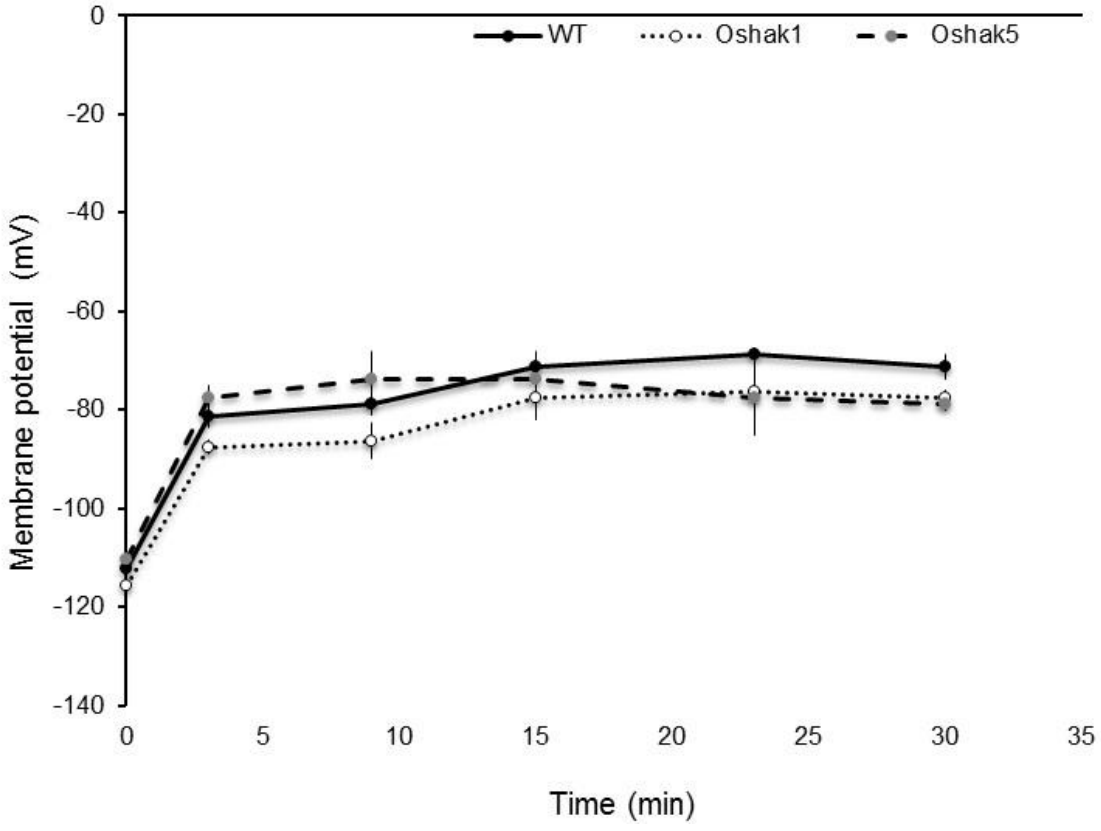

Figure 3. 12 Time dependence of membrane potential of wild type (DJ), *Oshak1* and *Oshak5* mutants after addition of 40 mM NaCl, measured in the mature zone of 5-6-day-old seedlings. Data are the mean ± SE (n=6).

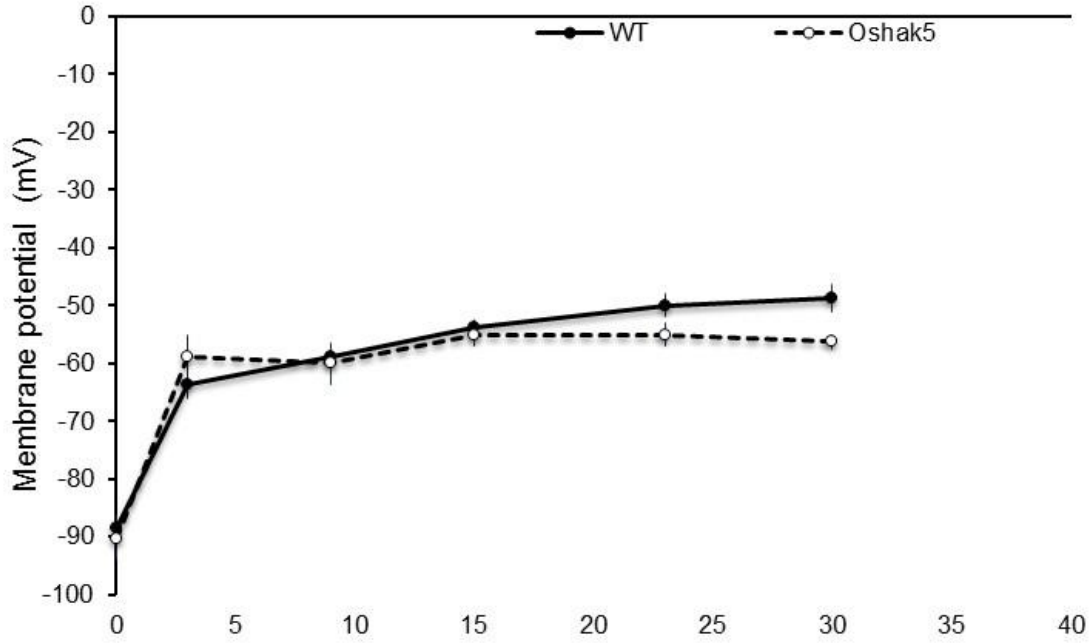

Figure 3. 13 Time dependence of membrane potential of wild type (HY), and *Oshak5* mutant after addition of 40 mM NaCl, measured in the mature zone of 5-6-day-old seedlings. Data are the mean ± SE (n=6).

3.4 Discussion

Potassium is an essential nutrient that affects a number of key physiological and metabolic processes responsible for plant growth and development. The high-affinity K^+ uptake system including the *HAK1* and *HAK5* genes, also plays an important role in K^+ nutrition, particularly under conditions of K^+ deficiency and salinity (Gierth et al., 2005; Nieves-Cordones et al., 2010; Santa-María et al., 1997).

Yang et al. (2014) and Chen et al. (2015) found that loss of function of the *Oshak1* and *Oshak5* genes resulted in a more significant growth impairment in mutants than in the wild type, under saline conditions. This difference was as high as 60%, under K^+ deficiency.

A plant's ability to maintain a high cytosolic K^+:Na^+ ratio is critical for the survival under salt stress (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008). In support of this, the present study showed that the shoot K^+ concentration (Table 3.6) was not significantly different between the *Oshak5* mutants and the wild types under saline condition, only the *Oshak1* mutants have lower K^+ when compared to the wild type plants, under saline stress (Table 3.7). Furthermore, the loss of function of *Oshak1* and *Oshak5* increased the shoot Na^+ concentration as compared to their wild types plants under salt stress (Table 3.6, 7). This resulted in root and shoot K^+:Na^+ ratios that were lower in the mutants than in the wild types (Table 3.6,7), thereby increasing the salt sensitivity of the latter. Additionally, plant growth and salt tolerance of the *Oshak1* mutants were significantly reduced when the mutants were grown in salt and K^+-deficient conditions (Table 3.5). Consistent with these results, Nieves-Cordones et al. (2010) reported that AtHAK5 is required to maintain plant growth under K^+-deficient as well as normal and saline conditions in Arabidopsis. This suggests that HAK5 plays a major role in K^+ acquisition, under low K^+ conditions. Santa-María et al. (1997) also reported that barley HvHAK1 conferred high-affinity K^+ uptake, and this is important for plant K^+ nutrition and Na^+ tolerance. These results provide further evidence of the

crucial role of *Oshak1* and *Oshak5* in plant salt tolerance, particularly under conditions of low external K^+ concentration.

The growth impairment noted in the *Oshak1* and *Oshak5* mutants, under the saline and the K^+ deficient condition, may have occurred for several reasons. Firstly, a reduction in the cytosolic K^+ concentration alters the activation of cytosolic enzymes, and lowers the plant's ability to maintain the optimum cytosolic pH (Hawkesford et al., 2012). This change in enzyme activity leads to an increase in soluble carbohydrates, especially reducing sugar, and soluble organic N compounds (Armengaud et al., 2009). Secondly, potassium is a major inorganic osmolyte that on average contributes to between 35 and 50% of the overall osmotic potential of plant cells (Shabala and Pottosin, 2014). Therefore, in the present study, a failure of the mutants to maintain adequate cytosolic K^+ concentration due to a loss of function of *Oshak1* and *Oshak5* imposes certain metabolic costs; these include the consumption of a large proportion of the plant's energy because of its need to generate organic solutes for osmotic adjustment. On the other hand, it was noted that the mutants showed a higher degree of shoot Na^+ accumulation than the WT. Thus, it is possible that the mutants intended to use Na^+, as an osmoticum, to compensate for their inability to acquire K^+ for osmotic adjustment. Thirdly, K^+ is required for cell extension, because it stabilises the pH in the apoplast and cytoplasm, and for the maintenance of the vacuolar osmotic potential. Consequently, as was found in the study by Hawkesford et al. (2012) a reduction in cytosolic K^+ concentration significantly reduced turgor, cell size and leaf area of plants.

3.4.1 Salinity resulted in K^+ efflux in rice plants

Adding 40 mM NaCl to the measuring bath resulted in a significant K^+ efflux, in both the elongation (Fig 3.1) and mature zones (Fig 3.2) of the wild types, and the *Oshak1* and *Oshak5* mutants, with the only exception being in the elongation zone of the wild type, where the K^+ efflux reduced, relative to the control. Several reasons for this K^+ efflux under saline conditions have been proposed. Firstly, because of the similarity between the electro-chemical features of

potassium and sodium, the latter can directly compete with potassium for binding sites in the plasma membrane including low-affinity potassium, such as NSCCs, or high-affinity K^+ uptake ones, such as HKT (Shabala and Pottosin, 2014). As the MIFE technique measures net ion flux, the reduced unidirectional K^+ uptake may shift the overall flux towards net efflux. Secondly, the Na^+ uptake through the plasma membrane causes significant membrane depolarisation, allowing K^+ efflux through the outward-rectifying depolarisation-activated K^+ channel, GORK (Shabala and Cuin, 2008). Thirdly, a toxic level of cytosolic Na^+ sets of a chain reaction: it causes a rise in concentration of cytosolic Ca^{2+}, thereby increasing H_2O_2 production, which, in turn, activates both GORK (Demidchik et al., 2010) and NSCCs (Demidchik et al., 2003), resulting in further K^+ efflux. If plants are able to control the efflux pathway, or compensate for the loss of K^+, by up-regulating the genes responsible for K^+ uptake, then the plants are able to complete their life cycle, or at least survive, under saline conditions.

3.4.2 The loss of function of *Oshak1* and *Oshak5* under conditions of K^+-deficiency, reduced the rate of K^+ influx

Yang et al. (2014) reported that *OsHAK5* expression was prominent in the root epidermis and parenchyma of stele tissues. After the removal of K^+ from the growth medium, the expression of *OsHAK5* increased, transiently, by about 10-fold at 1h and 3-fold, at 6h. On the other hand, salinity up-regulated the transcript levels of *OsHAK1*, regardless of the external K^+ concentration (Chen et al., 2015), and also up-regulated the transcript level of *OsHAK5* in the rice roots, by about 2-fold after 1h of treatment (Yang et al., 2014). In view of these findings, it is remarkable that upon salt exposure, the K^+ efflux was reduced in the elongation zone of the wild type compared to the control (Fig 3.1a). Various studies have concluded that salinity increases cytosolic Ca^{2+} concentration (Jiang et al., 2013; Kader and Lindberg, 2010; Kurusu et al., 2015; Laohavisit et al., 2013). This increased concentration plays a key role in stress signaling. Overall, the results of the present study indicate that the expression of *OsHAK1* and *OsHAK5* may be up-regulated in the

wild type, but only under low K^+ conditions, resulting in a decrease in K^+ efflux. A recent study by Ragel et al. (2015) has provided evidence that Ca^{2+} sensors, calcineurin B-like CBL1, 8, 9, 10 interacting protein kinase 23, (CIPK23) regulate the post-transcription of the HAK5 transporter by phosphorylation, the result being an increase in K^+ uptake in plants.

On the other hand, results of the present study found that the loss of function of *Oshak1* and *Oshak5*, resulted in an immediate K^+ efflux, which was 3-4 times higher than it was in the wild type under the high-affinity potassium condition (Fig 3.1a). Chen et al. (2015) reported that in the presence of a low external K^+ concentration, OsHAK1 contributed to about 50-55% of high-affinity K^+ uptake, during short-term rice plant growth. However, during long-term rice growth, its contribution was about 80, and 60%, of the total K^+ uptake, with low, and high external K^+ concentrations, respectively. The loss of function of *Oshak1* reduced the mean K^+ uptake rate of the mutant, by 50%, and 70% of the rate of the wild type grown in 0.1, and 1 mM K, respectively. Under low K^+ conditions, adding 50 mM NaCl largely prevented the uptake of K^+, particularly in the *Oshak1* mutant, suggesting the importance of its role in plant salt tolerance. Another study found that loss of function of *Oshak5* under K^+-deficient conditions caused a reduction of 80% in net K^+ uptake compared to the wild type. However, increasing the external K^+ concentration greatly stimulated the net K^+ influx, thereby reducing the difference between the wild type and the *Oshak5* mutant by 15% (Yang et al., 2014).

Results of the present study showed that the difference between the mutants and the wild type was almost non-existent under low-affinity potassium uptake conditions, while this difference was significant under conditions of high-affinity potassium. This confirms that OsHAK1 and OsHAK5 are K^+ transporters under a high-affinity potassium system (Fig 3.1a, c). Under high-affinity K^+ uptake, K^+ influx is consistently mediated by transporters, such as HAK, while under low-affinity K^+ uptake, the K^+ influx is passively mediated by channels (Alemán et al., 2011; Maathuis and Sanders, 1996). These can thermodynamically catalyse downhill fluxes that are three

orders higher than those catalysed by pumps and transporters (Tester, 1990). The activity of such channels relies on the electrochemical potential gradient of K^+ (Britto and Kronzucker, 2008). According to Chen et al (2015) *OsHAK1* was expressed particularly in the epidermal and vascular cells of rice roots where its expression was much stronger in both the tip of the preliminary root, and in the lateral roots. Accordingly, the results of the present study demonstrated a clear and significant difference in the way OsHAK1 and OsHAK5 function in the elongation zone compared to the mature zone (Fig 3.1, 3.2).

3.4.3 Salinity induced H^+-ATPase activation differs, according to the type of affinity potassium system, and genotype

Salinity causes membrane depolarization (Fig 3.12, 13). In order to restore MP, plants need to activate electrogenic plasma membrane H^+-ATPase, which transports H^+ from the cytoplasm of the cell to the external medium, creating electric gradient at the plasma membrane. Increased H^+-ATPase activity may provide a further driving force either for potassium uptake via voltage-gated channels, or for high-affinity K^+ uptake by HAK/KUP transporters (Shabala and Pottosin, 2014). Another beneficial aspect of H^+-ATPase activation is the exclusion of Na^+ by SOS1 Na^+/H^+ exchanger (Maughan et al., 2009). Altogether, these functions may enhance overall plant salt tolerance (Bose et al., 2015). Surprisingly, the present study's results showed a higher H^+ efflux from the elongation and mature zones of *Oshak1* and *Oshak5* mutants compared to the wild type (DJ), but only under low K^+ conditions. One possible explanation for this is that a loss of function of *Oshak1* and *Oshak5* may increase a plant's reliance on Na^+ as an omoticum (under conditions of reduced K^+ uptake), particularly in a high-affinity uptake range. Thus, H^+-ATPase activity increased significantly to energies a plasma membrane Na^+/H^+ exchanger, such as SOS1 in order to detoxify, by removing the excess amount of cytosolic Na^+, while maintaining a functional K^+:Na^+ ratio in plant roots. Further support for this explanation comes from the fact that H^+ efflux, in the wild type (DJ), gradually increased over the time, but at a lower rate than in the mutants.

Such a difference was not evident between the *Oshak5* mutant and the wild type (HY) in both of which the H^+ efflux dramatically increased. Another possible explanation is that *HAK* genes are K^+/H^+ symporters (Grabov, 2007), thus the rate of H^+ efflux was lower in the wild type (DJ) than in the mutant, as the H^+ had been used by OsHAK1 and OsHAK5 for K^+ uptake, while the higher efflux reading was due to the loss of function of the *Oshak1* and *Oshak5*. Furthermore, the results showed that the opposite trend occurred, under the low-affinity potassium system. In this case, different type of genes were functioning in K^+ uptake under this condition, so while H^+ was not needed to increase the rate of K^+ uptake by *OsHAK1* and *OsHAK5* genes, it was needed to activate the Na^+/H^+ exchanger to reduce the cytosolic Na^+ concentration. Thus, H^+ efflux was higher in the wild type (DJ) than in the mutants, in the elongation zone (Fig 3.3a), and in the *Oshak5* mutant than in the wild type (HY), in mature zone (Fig 3.4c).

3.4.4 The loss of function of *Oshak1* and *Oshak5* reduced the expression level of most of *OsRbohs* genes, resulting in lower K^+ efflux in the elongation zone under the high-affinity potassium system

Root exposure to exogenous ROS results in a higher K^+ efflux and Ca^{2+} influx, via NSCCs (Demidchik et al., 2003). Accordingly, it was anticipated that adding 10 mM H_2O_2 to the measuring medium would cause greater K^+ efflux in the *Oshak1* and *Oshak5* mutants than in the wild type, due to the inability of these mutants to compensate for the K^+ efflux that is caused by oxidative stress. Surprisingly, K^+ efflux was significantly higher in the elongation zone of the wild type compared to that of the mutants, reaching a peak after 25-30min (Fig 3.5). In order to interpret these results, further investigation was done on the expression levels of the respiratory burst oxidase homolog *OsRbohs* genes under the low K^+ condition. The results showed that the expression levels of *OsRbohA, B,* and *C* were 7-14 times higher in the wild type than in either the *Oshak1 or the Oshak5* mutants, while the expression level of *OsRbohG* was lower in the *Oshak5* (DJ) and higher in the *Oshak5* (HY) mutant as compared to the expression level of *RbohG* in the

wild type, suggesting that loss of function of *Oshak1* and *Oshak5* down-regulated the expression level of *OsRbohs* (except of *RbohG* in the *Oshak5* (HY) mutant plants), under the low K^+ condition (Fig 3.11). Consequently, the addition of 10 mM H_2O_2 together with higher *OsRbohA, B, C,* and *G* expressions (except of *RbohG* in the *Oshak5* (HY) mutant plants) caused a greater accumulation of ROS in wild type compared to the mutants, in the elongation zone. This, in turn, resulted in a strong signal that increased the K^+ efflux via NSCCs and GORK (Shabala and Pottosin, 2014).

Various studies have established that Rbohs proteins are synergistically activated by the binding of calcium to the Rboh EF-hand motifs, as well as by Ca^{2+} dependent phosphorylation. Once activated, these proteins promote ROS production, which subsequently activates Ca^{2+} channels (Kurusu et al., 2015; Wong et al., 2007).

The expression of the various isoforms of Rbohs proteins, which varies in response to different stimuli, caused varying levels of ROS production in plant tissues and cell organs. This, in turn, regulates the biological effects of ROS in that particular site of action (Rovira and Finkel, 2008). The results of this experiment showed that each genotype had a different K^+ flux profile: adding 10 mM H_2O_2 increased K^+ efflux by equal amounts in both the wild type and the *Oshak1* mutant. However, over the time, the K^+ efflux in the *Oshak1* mutant decreased significantly than in the wild type (DJ). This was the same trend displayed by K^+ efflux in the elongation zone (Fig 3.5a). In contrast, a dramatic and significant K^+ efflux occurred in the mature zone of *Oshak5* compared to the wild type, suggesting that a possible defect in the K^+ uptake function, (Fig 3.5c) in the mature zone of the *Oshak5* mutant, makes the plant unable to compensate for the K^+ efflux that results from oxidative stress.

Under low-affinity potassium conditions, no significant difference was observed between the wild type (DJ) and the *Oshak1* and *Oshak5* mutants, in terms of K^+ flux (Fig 3.6b, 7b), while K^+ efflux, both in the mature and elongation zones was significantly higher for the wild type (HY) than for the *Oshak5* mutant (Fig 3.6d,7d).

3.4.5 The loss of function of *Oshak1* and *Oshak5* may indirectly affect cytosolic Ca^{2+} concentrations impacting cell extension and root growth

The early signaling response, within seconds or minutes, to a biotic stress includes an increase in Ca^{2+} influx into the cytosol (Demidchik et al., 2007), which, in turn, activates Rbohs-dependent ROS production. This is followed by an amplification loop between Ca^{2+} and RbohC to regulate other biological processes of plant growth and development. Results obtained in the present study consistently demonstrated that the addition of 10 mM H_2O_2 increased Ca^{2+} influx in the elongation zone (Fig 3.9), and that such influx was significantly higher in the wild type than in the *Oshak1* and *Oshak5* mutants. This suggests that the high cytosolic Ca^{2+} concentration of wild type-activated *OsRbohs* genes caused an over-accumulation of ROS, which in turn, resulted in a further increase in K^+ efflux. Demidchik et al. (2002) reported that the greatest Ca^{2+} uptake for root growth occurs in the elongation zone, and, that a raised concentration of cytosolic Ca^{2+} stimulates exocytosis (Carroll et al., 1998), which supports elongation of cells in the roots (Cramer and Jones, 1996).

Foreman et al. (2003) showed that ROS production gradually increased in the wild type root hair during the process of its emergence from an initial bulge to an elongated tip, over a 1h period. In contrast, there was no ROS accumulation in the *rhd2* root-hair bulge over the same time. This suggests that loss of function of *rhd2* enabled the plant to produce ROS, which is required to elevate the Ca^{2+} gradient in the tips of root hairs. The study concluded that ROS produced by the activity of RHD2, is required to stimulate Ca^{2+} influx, during hair elongation and cell extension. In accordance with these findings. The results of the present study show that there is a link between higher levels of *OsRbohA, B, C* and *OsRbohG* expression in the wild type (DJ) and the higher levels of *OsRbohA, B, C* expression the wild type (HY) and the activation of hyperpolarisation-activated Ca^{2+} channels. These might facilitate the Ca^{2+} influx required for cell extension, in the elongation zone, under high-affinity potassium uptake conditions. This explains why the loss of

function of *oshak1* and *oshak5*, particularly under high-affinity uptake conditions, down-regulated the expression level of *OsRbohs* (Fig 3.11), which suppressed ROS production, thereby lowering the Ca^{2+} influx that is required for cell extension and growth in the elongation zone.

To summarise, this study provides further evidence that OsHAK1 and OsHAK5 both play a crucial role in intracellular K^+ homeostasis, particularly under high-affinity potassium-uptake conditions, by modulating the expression level of OsRbohs proteins.

Chapter 4

The role of inward rectifying OsAKT1 potassium channel in K^+ and NH_4^+ nutrition of rice plants and their response to salinity

4.1 Introduction

Potassium is being an essential nutrient for plants and plays an important role in many plant metabolic processes, such as enzyme activation, protein synthesis, photosynthesis, osmoregulation, and stress tolerance (Marschner and Rengel, 2012). Therefore, an understanding of the mechanisms of K^+ uptake and transport in higher plants is essential for improving plant growth, particularly under such an adverse environmental condition as salinity, which is the primary focus of the present study. A number of studies have shown that K^+ can be transported from the soil via two systems that differ in their affinity to K^+. These are classified on the basis of the external concentration of K^+. The first, high-affinity potassium uptake system is an active mechanism operating within the micro-molar range and mediated by an H^+-coupled transporter from the *KT/KUP/HAK* family. The second, a low-affinity potassium uptake system is a passive mechanism which operates within the milli-molar range of external K^+ concentrations and is mediated by inward-rectifying channels such as AKT1 and KAT (Epstein, 1972; Epstein et al., 1963; Hirsch et al., 1998; Maathuis and Sanders, 1996; Maathuis and Sanders, 1997; Sentenac et al., 1992). However, this classic view is somewhat negated by the fact that AKT1 has also been shown to be involved in K^+ uptake from solutions that contain as little as 10 micro-molar potassium (Hirsch et al., 1998), which suggests that AKT1 may potentially mediate K^+ uptake both in the low and high-affinity range of concentrations. Several factors can affect the plant's ability to uptake potassium from the soil solution. One of these factors is the availability of K^+ in the soil solution. Since the potassium concentration in agricultural soils is typically rather low (between 100 and 500 µM; (Claassen et al., 2001), and the concentration of K^+ in the cytoplasm normally

lies between 100 and 200 mM, this implies that very little of the K^+ that is required by the plant for growth is available for uptake, at the soil-root interface. The physiological function of AKT1 in K^+ uptake, under low-potassium conditions has been the focus of studies over the last three decades. These studies have shown that AKT1 contributes to the K^+ permeability of the plasma membrane in apical root cells, and this permeability accounts for 50% in the mesophyll cells of leaves (Dennison et al., 2001). The AKT1 contribution to root K^+ permeability is reported to be between 55 and 63%, in the absence of NH_4^+, and when the external K^+ concentration is between 10 and 1000 µM, thus indicating that AKT1 channel is an important component of the K^+ uptake system for enhancing plant growth (Spalding et al., 1999). OsAKT1, a rice homologous to the Arabidopsis inward-rectifying potassium channel AKT1 ((Locus ID is At2g26650) is predominantly localised in the epidermis and endodermis of rice roots (Golldack et al., 2003) and is modulated by CBL1-CIPK23 complex (Li et al., 2014). The loss of function of *Osakt1* increased the sensitivity of the genotype under the low K^+ condition, where the *Osakt1* mutant was characterised by a low K^+ content, and lower plant growth and development compared to the wild type (Li et al., 2014). Similar observations were manifested by the *Oscipk23* mutant, suggesting that OsAKT1 mediates K^+ uptake, and is modulated by OsCIPK23 (Li et al., 2014). In a recent study by Ahmad et al. (2016), manipulation of *AKT1* expression was shown to alter K^+ uptake and tissue content. This study showed that the loss of function of *Osakt1* resulted in growth reduction in the rice mutants, while overexpression of *OsAKT1* caused higher leaf K^+ content as compared to the wild type. However, while overexpression of *OsAKT1* improved growth, this came at the cost of less K^+ use efficiency.

The second research question addressed in the present study is sodium, and in particular, its interaction with potassium, in varying combinations of concentrations. A study by Spalding et al. (1999) demonstrated that the permeability of K^+ was enhanced, under a low external K^+ concentration, and in the presence of Na^+. The result was that the growth of the *akt1* mutant

increased two times in the presence both of Na^+, and10 µM, but not when the K^+ was increased to 100 µM K^+. This result suggested that AKT1 did not play a role in Na^+ transport. However, AKT1 may mediate Na^+ uptake when the external Na^+:K^+ ratio is high (Amtmann and Sanders, 1998). On the other hand, numerous other studies have concluded that there is a negative correlation between high Na^+ concentration, and potassium in the growth medium. This is because a high Na^+ concentration reduces the availability of potassium in the soil solution for plant growth, and limits K^+ permeability by competing with the K^+ at the binding sites on the plasma membrane (Shabala and Pottosin, 2010). Moreover, salt stress induces depolarisation of the plasma membrane, which, in turn, makes passive K^+ uptake via inward-rectifying K^+ channels thermodynamically impossible. On the other hand, this depolarisation activates the outward- rectifying channels, causing further K^+ efflux (Shabala and Pottosin, 2010). A study of the role played by AKT1 in the salt tolerance of rice showed that the *AKT1* transcript was regulated differently in the different rice genotypes, when salt stress was induced, altering the plants' salt tolerance (Golldack et al., 2003).

The third research objective of the present study was to investigate how NH_4^+ influences K^+ uptake in rice plants. NH_4^+ is an essential nitrogen nutrient for plants. However, high concentrations of it can be toxic to plant growth, particularly under low K^+ conditions (ten Hoopen et al., 2010). Despite the fact that NH_4^+ and K^+ share similar features, such as charge, size and their effect on membrane electrical potential (Wang et al., 1996) some studies have identified a negative correlation between net K^+ flux and NH_4^+, whereby NH_4^+ reduces K^+ uptake and accumulation in rice (Szczerba et al., 2008a; Wang et al., 1996), Arabidopsis (Spalding et al., 1999), and barley (ten Hoopen et al., 2010). However, this effect can be ameliorated by increasing the K^+ concentration of the growth medium. It has been suggested that the influence of NH_4^+ is dependent on the external concentration of K^+. In the high-affinity K^+ range, K^+ uptake is mediated by NH_4^+-sensitive components, while in the low-affinity range, K^+ uptake is mediated by NH_4^+-

insensitive components (Santa-María et al., 2000; Szczerba et al., 2008b). A study on Arabidopsis demonstrated that NH_4^+ specifically inhibits the non-AKT1 component, by competing with K^+ on the binding sites on the transporters (Spalding et al., 1999), while another showed that NH_4^+ uptake can be mediated by plant K^+ transporters and channels (Wang et al., 1996). Globally, the use of mineral fertilisers to provide plant nutrients is expected to increase from 175 million tonnes in 2015 to 199 million tonnes by 2030 (Roy et al., 2006). Since such increased use of fertilisers will prove increasingly costly to the agricultural industry, a better understanding of the mechanisms involved in plant nutrition will make it possible for farmers to use lower quantities of fertilisers, more efficiently. As mentioned above, some studies showed that the uptake of NH_4^+, a main source of the essential element for plant growth is mediated by plant potassium transporters and channels, particularly under low K^+ conditions (ten Hoopen et al., 2010). On the other hand, the presence of a high-salt concentration changes the cytosolic concentration of K^+, and so impairs plant growth. Thus, by focusing on the inward-rectifying K^+ channel, OsAKT1, the interaction between three elements, Na^+, K^+ and NH_4^+ can be studied, and the way their uptake mechanisms function under adverse environmental conditions may be understood. Therefore, the present work aimed to study the effects of overexpression of *OsAKT1* gene in the rice mutant on Na^+, K^+ and NH_4^+ fluxes compared to those in the WT. Our data revealed that OsAKT1 mediates NH_4^+ uptake under low-K^+ conditions. Moreover, the inhibitory effect of a high NH_4^+ concentration was not only on the non-AKT1 component, but also on OsAKT1. This effect caused a significant reduction in the K^+ flux. Overexpression of *AKT1* enhanced uptake of both Na^+ and K^+ under low K^+ conditions.

4.2 Materials and Methods

4.2.1 [4]Plant materials

Seeds of *O. sativa L. Japonica* cv Nipponbare wild type, and its mutants, OsAKT1-OX1, OsAKT1-OX2, and *Osakt1* and those of *O. sativa L. Japonica* cv Dongjin wild type, and its mutant, *Osakt1* were obtained from Dr Frans Maathuis, Department of Biology, University of York, UK. Transposon and T-DNA insertion of *Osakt1* lines, analysis of the loss of function in mutants, as well as rice transformation and the validation of AKT1-OX transgenic lines are all fully explained in the supporting information section.

4.2.2 [5]Whole-plant physiological assessment

Rice seeds were geminated on terra green for 5 days in the dark at 28 °C and 90% relative humidity. Then, the seedlings were grown in a standard growth medium for three weeks before treatments were applied. The growth medium consists of macronutrients (2.9 mM NH_4NO_3, 0.3 mM NaH_2PO_4, 0.5 mM K_2SO_4, 1 mM $CaCl_2$, 1.6 mM $MgSO_4{\cdot}7H_2O$), micronutrients (Yoshida et al., 1976) and Na_2SiO_3 (0.18 g l^{-1}) and the pH was adjusted to 5.6-5.7. The growth medium was changed every 3 days.

K_2SO_4 in the standard medium was replaced with an equimolar quantity of Na_2SO_4 for the '0 K' condition. The salt stress of 60 mM NaCl was lasted for 2 weeks. To test the effect of ammonium, 3 mM NH_4^+ was added to the growth medium. Glass house conditions were: 16h light/8h dark; 28/24 °C day/night; 60% relative humidity with light radiation of about 160W m^{-2}. The relative growth rate (RGR) was calculated according to (Poorter and Garnier, 1996)

[4] Only one knockout mutant (*Osakt1*) was obtained for this study. The results of this study may be verified by generating RNAi plants in a future study

[5] Due to limited number of seeds available, all phenotyping experiments for this chapter were conducted by our collaborators at York University, UK, while our focus in Hobart was on electrophysiological studies.

4.2.3 Microelectrodes preparation

Microelectrodes preparation was carried out as described in the first experimental chapter. The electrode tip was front-filled with the corresponding LIX which listed in the following table (Table 4.1).

Table 4. 1 Ionophores (LIX) and the back-filling solutions which were used to prepare the microelectrode of the selected ion

Ion	Ionophore (LIX)	Back-filling solution (mM)
Na^+	N,N′,N″-Triheptyl-N,N′,N″-trimethyl-4,4′,4″-propylidynetris(3-oxabutyramide)	500 NaCl
K^+	Valinomycin	500 KCl

To calibrate the microelectrodes, an appropriate set of three standard solutions, (Table 4.2) covering the expected range of targeted ion, was prepared. A calibration of the microelectrodes was then performed as the procedure described in the first experimental chapter using the following calibration solutions.

Table 4. 2 Measured ions and their standard calibration solutions

Ion	Standard calibration solutions
Na^+	0.1, 0.5, 1.5, 10, 50, 100 mM NaCl
K^+	50, 250, 500, 1000 µM KCl
NH_4^+	20, 50, 100 and 500, 1000, 1500, 2000, 3000 µM NH_4Cl

4.2.3.1 Experimental protocols

Root preparation was carried out as described in the first experimental chapter. The growth media used for seed germination differed, depending on the particular objective of the research.

Distilled water, with either 20 µM or 2 mM of NH_4^+ were used to study the role of OsAKT1 channel in Na^+ uptake and Yoshida solution were used to study the role of OsAKT1 channel in NH_4^+ uptake. Yoshida solution consists of NH_4NO_3 (1.43 mM), $NaH_2PO_4.2H_2O$ (0.37 mM), K_2SO_4 (0.5 mM), $CaCl_2$ (1 mM), and $MgSO_4.7H_2O$ (1.6 mM). The pH level of the nutrient solution pH was maintained between 5-6 using sulfuric acid (Yoshida et al., 1976).

The BSM bathing medium consisted of 200 µM NaCl, 100 µM $CaCl_2$, and 200 µM KCl. To test the hypothesis that OsAKT1 functions in high-affinity potassium conditions, KCl concentration was reduced to 50 µM for the NaCl treatment under either 20 µM or 2 mM of NH_4Cl conditions. To study the effect of NH_4 on the functionality of OsAKT1, 20 µM of NH_4Cl was added to the BSM for the NH_4Cl and KCl treatments. The pH level of the BSM solution was around 5.4-5.5. For conditioning, roots were left in the bathing solution for approximately 30-60 min.

The measurement was carried out as the procedure described in the first experimental chapter. The treatments of 1 mM KCl, 1 mM NH_4^+, 1 mM NaCl, and 40 mM NaCl were used to achieve the aim of this study. The ion flux was measured in the elongation zone of rice root.

4.3 Results

4.3.1 The OsAKT1-K^+- NH_4^+ interaction

It has been reported that in the presence of NH_4^+ and under a low K^+ condition, the loss of function of *akt1* caused a growth reduction in Arabidopsis (Spalding et al., 1999). Thus, the present study tested the role of OsAKT1 in rice plants grown under these same conditions. The results showed no significant difference (Table 4.3) in the relative growth rate (RGR) between the *Osakt1* knock-out (KO) mutant and the wild type, either under the control condition or in the presence of NH_4^+. Therefore, the presence of NH_4^+ did not have an inhibitory effect on the growth of the *akt1* KO mutant in rice.

Table 4. 3 The effect of ammonium on the relative growth rate (RGR % day^{-1})) of 5-week-old rice plants of wild type (Nipponbare), and *akt1* both grown in a standard medium, and media containing 0K, and 3 mM NH_4^+, respectively.

Genotypes	Control	0K+3NH_4
	RGR (% day^{-1})	
WT (DJ)	16.5±1.6	7.5±0.8
Akt1 (DJ)	17.1±1.1	4.8±1.1

4.3.2 The role of OsAKT1 in rice salt tolerance

Earlier study has reported that salt stress regulates the expression level of *OsAKT1* in a highly specific genotype-dependent manner (Golldack et al., 2003). While *OsAKT1* transcript levels were diminished in salt tolerant rice genotypes, they remained stable in the salt sensitive ones, resulting in a high shoot Na^+ concentration (Golldack et al., 2003). On the other hand, other studies have shown that the expression level of *OsAKT1* is down-regulated in response to salt stress (Fuchs et al., 2005; Kavitha et al., 2012). In the light of these conflicting findings, the role of OsAKT1 in salt tolerance in rice was tested in this work using a K^+-free growth solution. The

results showed no significant difference in RGR, between the genotypes at 60 mM NaCl (Table 4.4).

Table 4. 4 The effect of salt stress on the relative growth rate (RGR) of 5-week-old rice plants of wild type (Nipponbare), *AKT1* overexpressors (OX1, OX2), and knockout *akt1* mutant grown in a standard medium, and a medium containing 60 mM Na^+.

Genotypes	Control	60 mM Na
	RGR (% day^{-1})	
WT (NB)	17.6±1.6	4.43±1.3
AKT1-OX1	16.3±0.6	5.21±0.5
AKT1-OX2	16.3±1.9	5.34±1.3
akt1	16.6±3.9	3.91±3.6

Interestingly, the salt stress significantly increased root Na^+ concentration in the *akt1* KO genotype, while slightly decreasing it in the overexpressing *AKT1* genotypes (Table 4.5). Shoot Na^+ content was similar in all genotypes, under the salt condition. When the genotypes were grown under the $0K^+$ condition, the overexpression of *OsAKT1* caused a significant increase in the root Na^+ concentration and, correspondingly a significant decrease in that of the *akt1* KO mutant (Table 4.5).

Table 4.5 Shoot and root Na^+ content of 5-week-old rice plants of wild type (NB), overexpressors (OX1, OX2), and *akt1*, when exposed either to OK or 60 mM NaCl. Significant difference between each genotype and its wild type, determined by t-test at a probability level of $P \leq 0.05$.

Na^+ content (µmol g^{-1} DW)				
	Root		Shoot	
Genotypes	0 K	60 mM Na	0 K	60 Mm Na
WT (NB)	0.15±0.02	0.85±0.1	0.26±0.09	1.41±0.09
AKT1-OX1	0.23±0.01	0.6±0.18	0.18±0.03	1.10±0.19
AKT1-OX2	0.28±0.04*	0.5±0.15	0.16±0.11	0.90±0.18
akt1	0.08±0.02*	1.8±0.17*	0.41±0.11	1.18±0.36

4.3.3 K^+ stimulates a short-term NH_4^+ influx

To identify the interaction between NH_4^+ and K^+ in the epidermal cells of the root surface, net NH_4^+ and K^+ fluxes were measured in the elongation zone of the overexpressor and KO lines, and the wild types. The seedlings were grown in Yoshida solution for 5 days, and then subjected to 1 mM KCl in a bathing medium containing low potassium (200 µM), and low NH_4^+ (20µM). The data showed that adding 1 mM KCl resulted in an increase in the NH_4^+ influx in the *OsAKT1* overexpressing line compared to the wild type (Fig. 4.1a); the NH_4^+ efflux turned into immediate influx upon treatment, and then gradually decreased, reaching -18 nmol m^{-2} s^{-1} after 25 min of treatment. This was above the basal level of the control (-54 nmol m^{-2} s^{-1}). In the wild type, the NH_4^+ flux increased immediately, but then decreased more significantly than in the overexpressor, reaching -117 nmol m^{-2} s^{-1} after 24 min of treatment. This was slightly higher than the basal level of the control (-106 nmol m^{-2} s^{-1}). In contrast, the *akt1* KO mutant, under the same condition (Fig. 4.1b), exhibited no response to the treatment, while an immediate lower efflux in the wild type occurred in response to the K^+ increment.

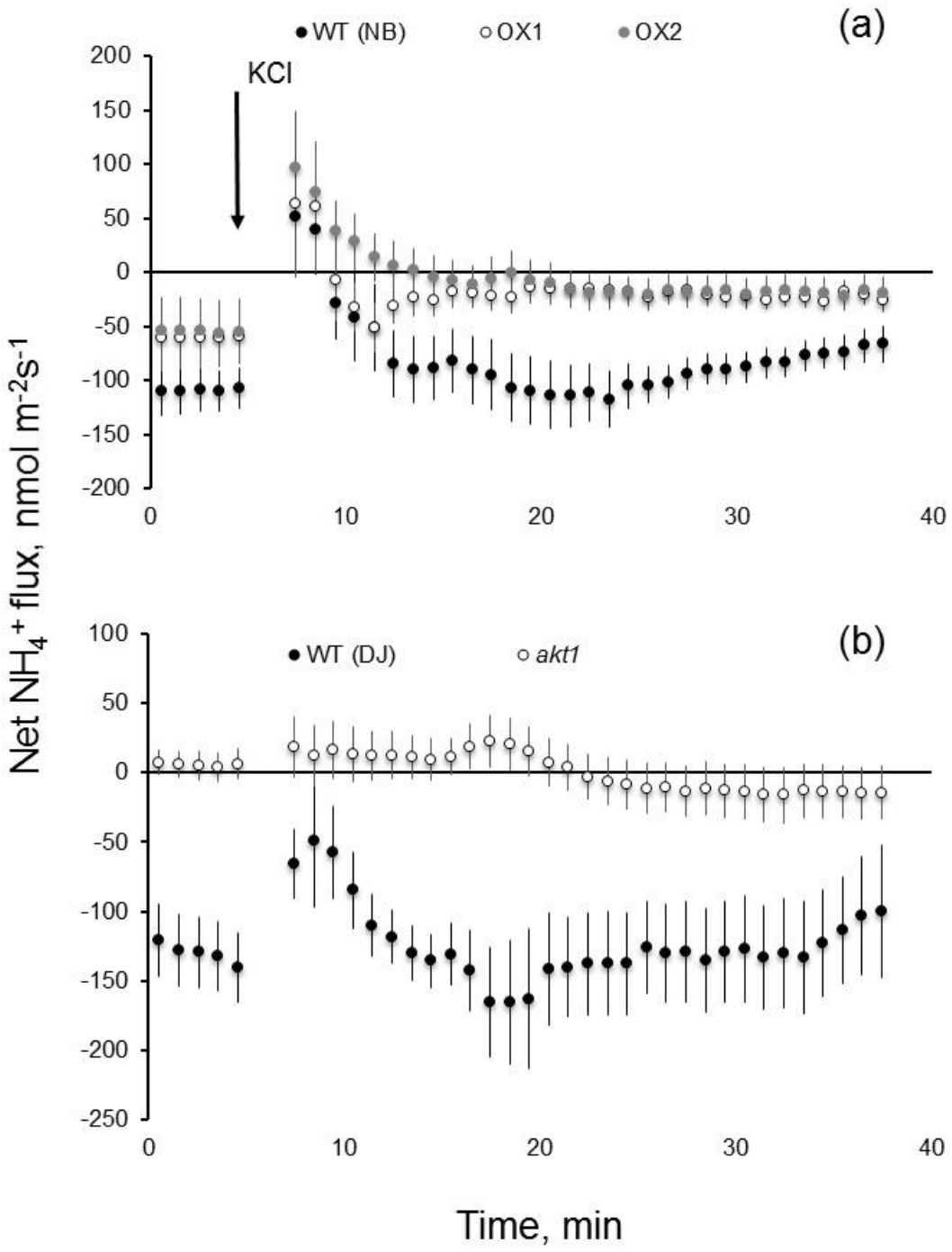

Figure 4. 1 Net NH_4^+ flux in the elongation zone of the root of the overexpressors OX1 and OX2 (a), *akt1* mutant (b), and their respective wild types. Rice seeds of all genotypes were germinated and grown in Yoshida solution, containing 1.5 mM NH_4^+ for 5-6-days. The rice seedlings were treated with 1mM KCl for 30 minutes. Data are the mean ± SE (n=6)

Under low potassium conditions, the addition of 1mM KCl resulted in no significant difference in the K^+ flux, response among all genotypes, (Fig. 4.2 a, b): all responded with an

immediate K^+ uptake, which, then, gradually decreased until it reached the basal level of the control. Overall, adding 1 mM of K^+ stimulated a NH_4^+ influx in the elongation zone of rice roots, particularly in that of the *OsAKT1* overexpressors, under low K^+ and NH_4^+ conditions, thereby revealing the physiological role of OsAKT1 in NH_4^+ uptake under varying K^+ conditions.

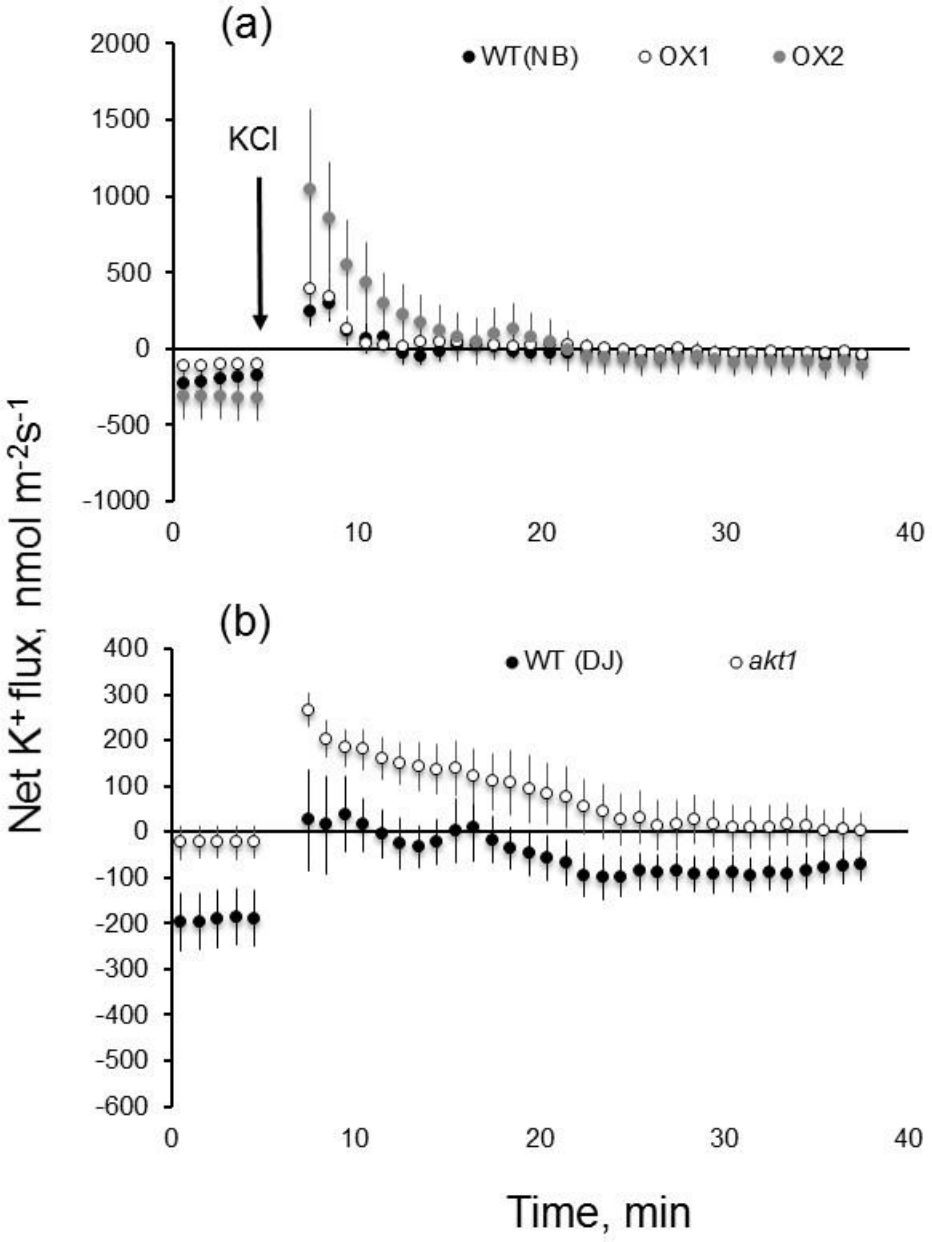

Figure 4. 2 Net K^+ flux in the elongation zone of the root of the overexpressors OX1 and OX2 (a), *akt1* mutant (b) and their respective wild types. Rice seeds of all genotypes were germinated and grown in Yoshida solution, containing 1.5 mM NH_4^+ for 5-6-days. The rice seedlings were treated with 1 mM KCl for 30 minutes. Data are the mean ± SE (n=6).

4.3.4 OsAKT1 plays no role in NH_4^+ uptake under NaCl treatment

The addition of 1 mM NaCl to the bathing medium did not reveal any OsAKT1 involvement in NH_4^+ flux in the presence of either NH_4^+ concentration (Fig. 4.3a.b.c.d). At the same time, upon exposure to 1mM Na^+, while the net NH_4^+ flux remained almost constant until the end of the measurement phase (Fig. 4.3a.c), salt stress caused a gradual NH_4^+ efflux that was non-significantly different in any of the genotypes, under either of the NH_4^+ conditions(Fig. 4.3b.d).

The difference in the ion flux in the OsAKT1 lines before the NaCl treatment was due to the fact that the number of seeds used were insufficient to standardise the reading. Normally, the main root of the individual seedling is selected for ion flux measuring; however in the case of the AKT1 lines, 4 roots were selected from the same seedling to perform the measurement. This may provide the main explanation for the significant variation in ion flux within the same line.

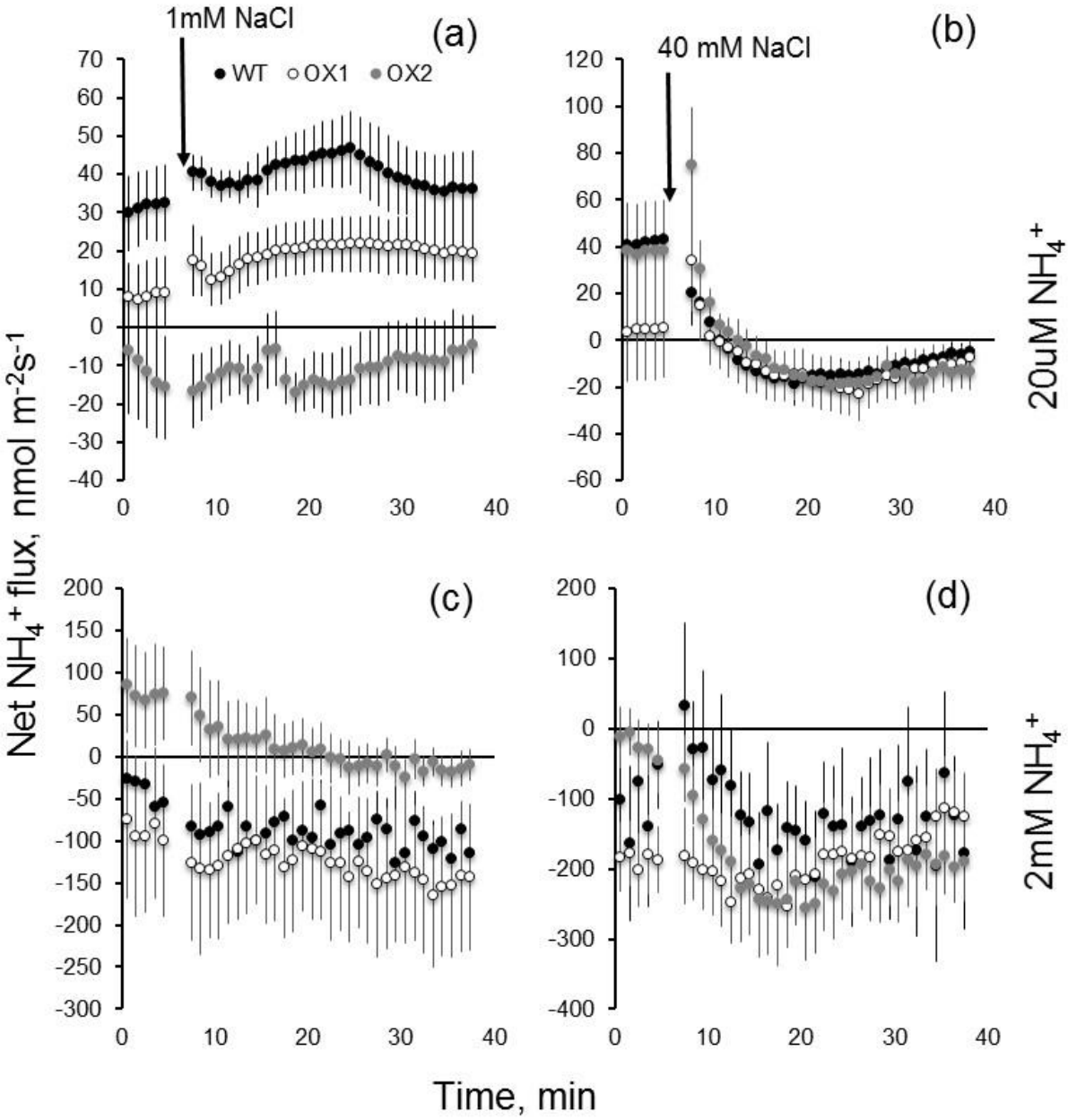

Figure 4. 3 Net NH_4^+ flux in the elongation zone of the root of the overexpressors OX1 and OX2, and their respective wild types. Rice seeds of all genotypes were germinated and grown in a solution containing either 20 µM, or 2 mM NH_4^+ for 5-6-days. The NH_4^+ flux was measured during the first 5 minutes before treatment, and then during the 30 minutes of treatment with either 1mM or 40 mM of NaCl in media containing either 20µM or 2 mM NH_4^+. Data are the mean ± SE (n=6).

4.3.5 Overexpression of *OsAKT1* enhanced net Na^+ uptake under both NH_4^+ conditions

The *OsAKT1* overexpressors had 2.5-3.5 times higher Na^+ uptake, and 4 times higher Na^+ efflux in the wild type than in control (Fig. 4.4a). Under salt stress conditions, the *OsAKT1*

overexpressor (OX1) showed a significantly higher Na^+ influx compared to the wild type (Fig. 4.4b). Thus, under the low NH_4^+ condition and both Na^+ conditions (1 and 40 mM NaCl), OsAKT1 mediates Na^+ uptake. This uptake was greater under 1 mM Na^+ condition. On the other hand, a high concentration of NH_4^+ in the bathing medium resulted in an OsAKT1 function that was exactly the opposite of that under the condition of low NH_4^+ concentration (Fig. 4.4c.d), where the overexpressors extruded more Na^+ than the wild type under 1 mM Na^+ condition, but with no significant difference between genotypes under salt stress conditions.

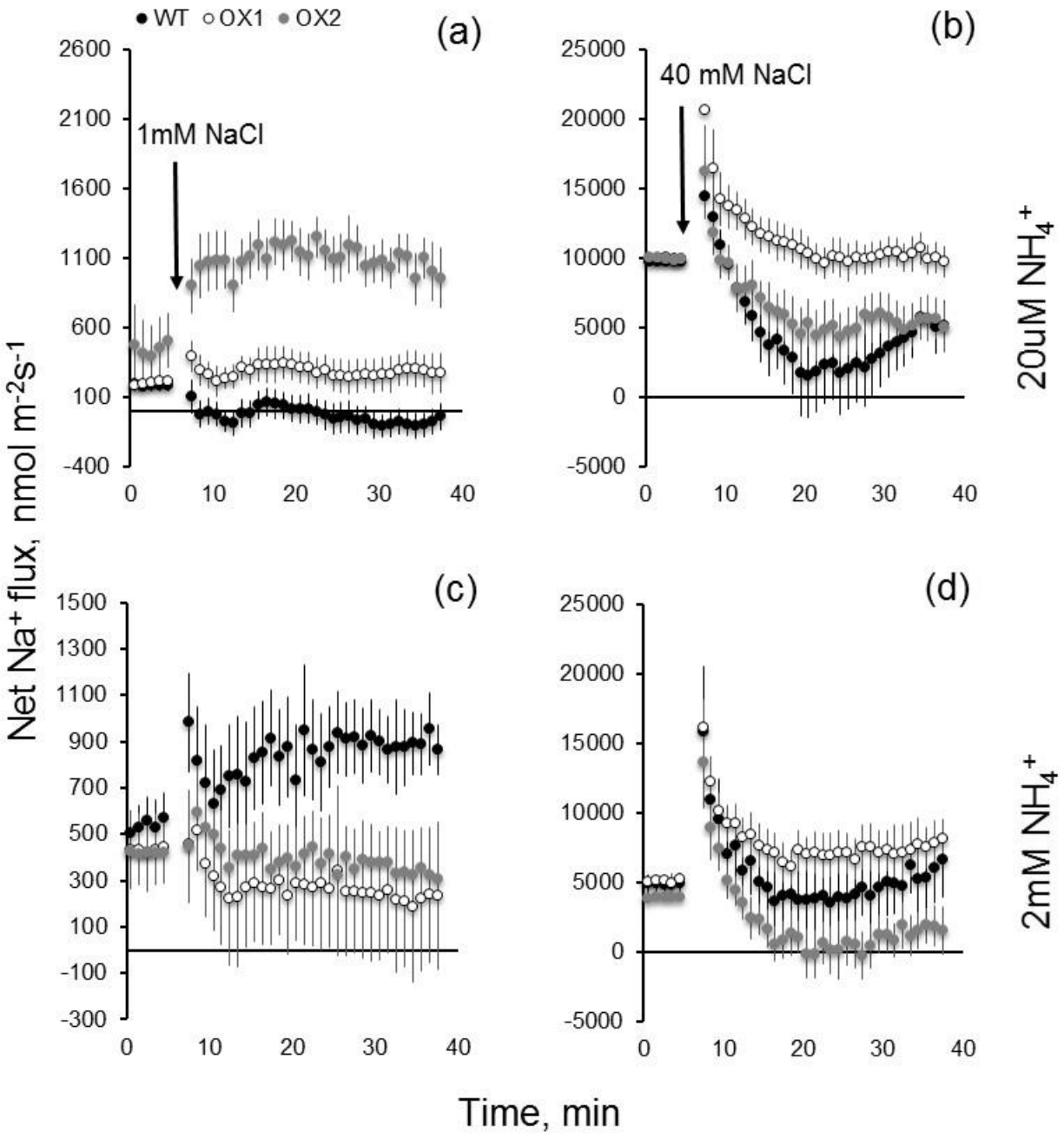

Figure 4. 4 Net Na^+ flux in the elongation zone of the root of the overexpressors OX1 and OX2, and their respective wild types. Rice seeds of all genotypes were germinated and grown in a solution contained either 20 µM, or 2 mM NH_4^+ for 5-6-days. The NH_4^+ flux was measured during the first 5 minutes before treatment, and then during the 30 minutes of treatment with either 1mM or 40 mM of NaCl in media containing either 20µM or 2 mM NH_4^+. Data are the mean ± SE (n=6).

4.3.6 Overexpression of *OsAKT1* enhanced K^+ uptake, under 1mM Na^+ treatment and low NH_4^+ condition

K^+ uptake in *OsAKT1* overexpressors gradually increased in response to the addition of 1 mM NaCl. However, in the wild type, the K^+ efflux was immediate, then gradually recovered until it reached the basal level. These results showed that OsAKT1 mediates K^+ uptake, both in the presence of a low NH_4^+ concentration and under1 mM Na^+ condition (Fig. 4.5a). There were no significant differences in the response of K^+ uptake to other treatments under either NH_4^+ conditions (Fig. 4.5b.c.d).

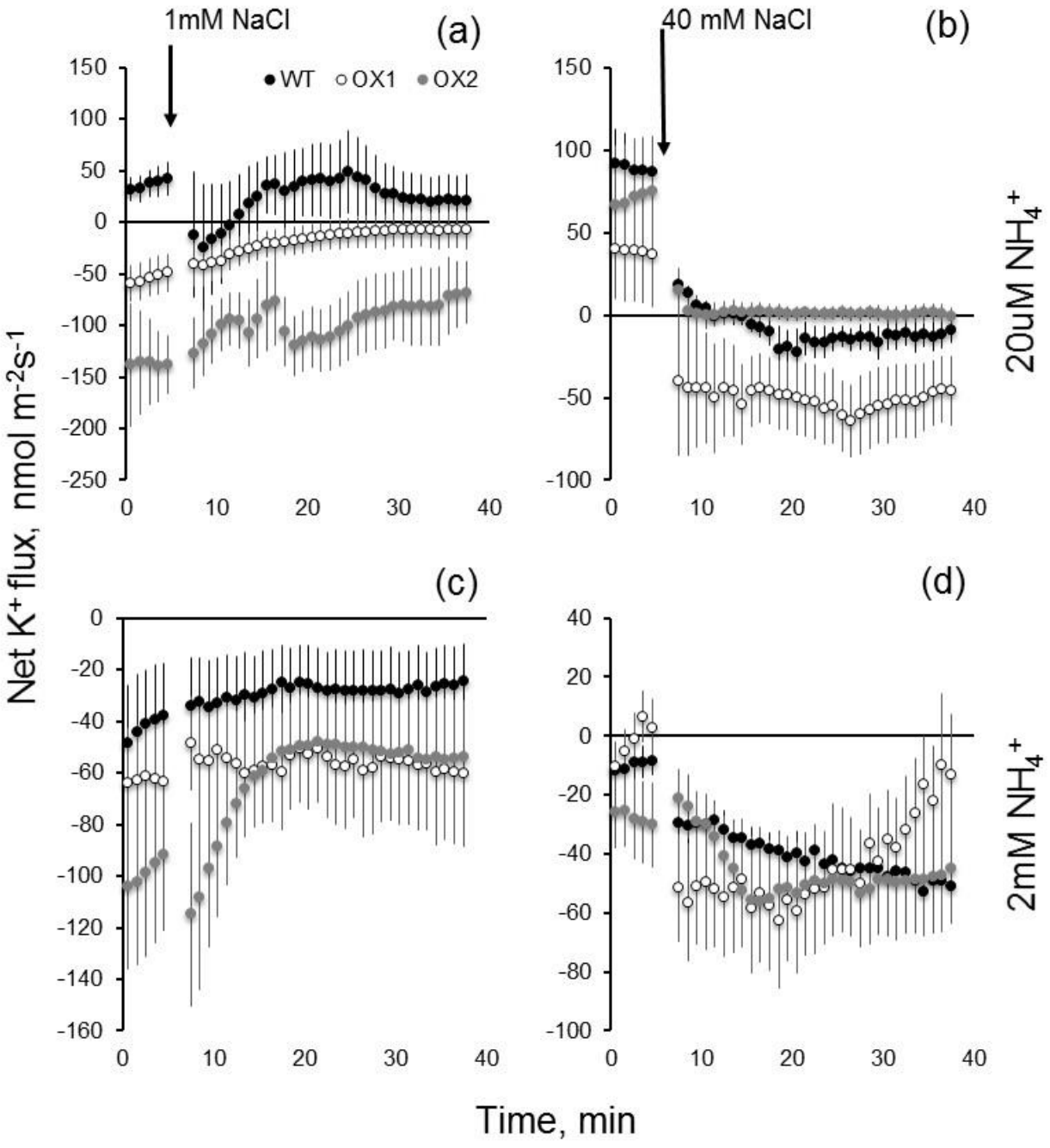

Figure 4. 5 Net K^+ flux at the elongation zone of the root of the overexpressors OX1 and OX2, and their respective wild types. Rice seeds of all genotypes were germinated and grown in a solution containing either 20 µM or 2 mM NH_4^+ for 5-6-days. The NH_4^+ flux was measured during the first 5 minutes before treatment, and then during the 30 minutes of treatment with either 1mM or 40 mM of NaCl in media containing either 20µM or 2 mM NH_4^+. Data are the mean ± SE (n=6).

4.4 Discussion

4.4.1 NH_4^+ influx was stimulated by increasing the extracellular K^+ concentration

A previous study of the competition between NH_4^+ and K^+ for uptake, in the root both of Arabidopsis and barley, found that the net NH_4^+ influx doubled when both K^+ and NH_4^+ were present in the growth medium compared to NH_4^+ alone (ten Hoopen et al., 2010). This suggests that K^+ stimulated NH_4^+ influx. Furthermore, to understand whether this influx was mediated by potassium channels or transporters, 10 TEA^+ and 0.1 Gd^{3+} were applied. The result was a significant reduction both in the NH_4^+ and K^+ influxes, supporting the notion that certain plasma membrane transporters or channels are involved in the transport of NH_4^+ and K^+ (ten Hoopen et al., 2010). In order to gain an understanding of the exact mechanism of this K^+ and NH_4^+ interaction, on the uptake site on rice roots, this study used the MIFE technique to measure NH_4^+ flux, in the *OsAKT1* overexpressors and *akt1* KO mutant, in response to the addition of K^+. Interestingly, the data showed that adding 1mM K^+ to the bathing medium stimulated stronger NH_4^+ influx in the elongation zone of *OsAKT1* overexpressors compared to the wild type (Fig 4.1a). On the other hand, loss of function of *akt1* in the *akt1* KO mutant resulted in no response to the K^+ treatment (Fig 4.1b). These results indicate that NH_4^+ influx may be mediated by the Shaker-type potassium channel OsAKT1, when the external K^+ concentration changed from HATS to LATS. This finding is in line with that of ten Hoopen et al. (2010) which showed that K^+ stimulated a short-term NH_4^+ influx in Arabidopsis plants. However, it contradicts the finding of Szczerba et al. (2008a) that NH_4^+ influx reduced by 60% and the cytosolic NH_4^+ by 3-4 times, when the external K^+ concentration changed from the HATS (0.1 mM) to LATS concentration range, and that it increased at 0.1 and 40 mM K^+, in the presence of TEA^+. This led to the conclusion that K^+-sensitive influx of NH_4^+ occurred, primarily, via the NSCCs pathway (Szczerba et al., 2008b). However, long term supplying of K^+ to barley roots, inhibited NH_4^+ accumulation (Szczerba et al., 2008a). Recently, Ahmad et al. (2016) reported that loss of function of *akt1* did

not alter NH_4^+ sensitivity. This conclusion was based on their finding of a similarity between the growth pattern of the *akt1* KO mutant and the wild type, an indication that the inhibitory action of NH_4^+ has a relatively short-term effect on flux assay, but not on long-term growth.

Our data also showed that in response to 1 mM K treatment, neither loss of function of *akt1* nor *OsAKT1* overexpression altered the K^+ influx under low NH_4^+ conditions compared to the wild type (Fig 4.2a, b). This result suggests that, both under high-affinity NH_4^+ and low-affinity K^+ conditions, OsAKT1 mediated NH_4^+, as a source of the N needed for plant growth.

4.4.2 Does OsAKT1 mediate Na^+ uptake?

A previous study found that when the plants were exposed to 150 mM NaCl, OsAKT1 transcripts in the exodermis of salt tolerant genotypes of rice diminished, with no change in those of the salt sensitive ones (Golldack et al., 2003). Furthermore, it was found that Na^+ exclusion in salt-tolerant genotypes depended on the external K^+ concentration, whereby they excluded Na^+ when this concentration was in the range of 10 µM to low-mM. However, under low potassium conditions, these genotypes accumulated similar quantities of Na^+ as the salt-sensitive ones (Golldack et al., 2003). This study suggested that OsAKT1 plays a role in Na^+ uptake, particularly under conditions of K^+ depletion. Based on this hypothesis, the present study measured the NH_4^+, Na^+, and K^+ fluxes in the elongation zone both of the overexpressors and the wild type, in order to study the role of OsAKT1 in Na^+ uptake, in the presence of two levels of NH_4^+ concentrations. Interestingly, results revealed that under the low NH_4^+ condition the addition of 1 and 40 mM NaCl to the bathing medium resulted in a higher Na^+ uptake in *OsAKT1* overexpressors than in the wild type (Fig 4.4). This suggests that OsAKT1 mediated Na^+ influx in response to 1 mM and severe sodium treatments both under low potassium and ammonium conditions. A previous study also showed that in response to a moderate saline condition, and under a low potassium condition, sodium uptake in red-beet was enhanced, with no influence either on plant water status or growth (Subbarao et al., 2000). This result suggested that Na^+ was needed for osmotic adjustment, in the

case of K^+ depletion (Subbarao et al., 2000). When the external K^+ concentration ranged between 10-100 µM, the presence of Na^+ doubled the permeability of K^+. This is in agreement with the finding on the growth rate of the *akt1* KO mutant, where the presence of Na^+ doubled its growth rate at 10 µM K^+, but not at 100µM K. This suggests that non-AKT1components may be involved in Na^+ uptake in the presence of a K^+ depleted concentration (Spalding et al., 1999). The results of the present study provide strong evidence that not only do non-AKT1 components mediate Na^+ uptake, but that OsAKT1 is also directly involved in the Na^+ uptake, when there is at low K^+ concentration, and with no competition from NH_4^+. Amtmann and Sanders (1998) suggested that although K^+ channels showed a higher permeability to K^+ rather than Na^+, they might mediate Na^+ transport under high salt stress conditions. The results of the present study confirmed that OsAKT1 does mediate Na^+ uptake both under normal and severe Na^+ conditions in the presence of a very low NH_4^+ concentration (Fig 4.4 a, b). The benefit of this for the plants is that the sodium is required as an osmoticum for osmotic adjustment under the low K^+ condition (Subbarao et al., 2000). It has also been reported, in studies on red-beets (Subbarao et al., 1999), olive trees (Erel et al., 2014), and wheat (Krishnasamy et al., 2014) that elements like Na^+ and K^+, can replace each other fully in certain non-specific metabolic functions of which osmotic adjustment is one.

4.4.3 Does NH_4^+ inhibit the Na^+ uptake that is mediated by OsAKT1?

The finding of the present study that OsAKT1 is involved in Na^+ uptake, under low NH_4^+ conditions raises a question of whether NH_4^+ has an inhibitory effect on Na^+ uptake that is mediated by OsAKT1. A previous study demonstrated that 2 mM NH_4^+ inhibited 50% of the non-AKT1 component, and 100% of it when the K^+ concentration is low (Spalding et al., 1999). The data of the present study showed that the presence of 2 mM NH_4^+ in the bathing medium resulted in no significant difference in Na^+ uptake, between the overexpressors and the wild type (Fig 4.4c, d). This finding suggests that NH_4^+ has an inhibitory effect not only on the non-AKT1 component, but also on OsAKT1 under low K^+ conditions. Thus, the *OsAKT1* overexpressor loses its capacity

to uptake Na^+ from the medium, and it showed a similar capacity for Na^+ uptake as the wild type. Using cowpea, Voigt et al. (2009) showed that the presence of 2.5, and 5 mM NH_4^+ in the medium, decreased Na^+ accumulation by 28%, and 52 %, respectively in the roots compared to the wild type growing in the presence, and absence of NH_4^+ respectively. This suggested that NH_4^+ inhibited Na^+ uptake in the roots of cowpea (Voigt et al., 2009). Based on its data, the present study concludes that OsAKT1 is involved in Na^+ uptake under the low NH_4^+ condition, and that the presence of high NH_4^+ inhibited the Na^+ permeability of OsAKT1 in the elongation zone of rice roots (Fig 4.4).

4.4.4 Overexpression of *OsAKT1* does not alter the NH_4^+ flux in response to Na^+ treatments

The NH_4^+ and Na^+ selectivity of DmKT1, the orthologous of AKT1, has been shown to be very low with no discrimination between them (Scherzer et al., 2015). Therefore, based on this finding, the present study anticipated that the overexpression of OsAKT1 would not make a significant difference to NH_4^+ flux in response to Na^+ treatments (Fig 4.3). The results did confirm this, indicating that the structure of this OsAKT1 channel may not account for the effects of Na^+ on NH_4^+ uptake in plant cells. This study concludes that OsAKT1 was insensitive to Na^+ treatments, in terms of NH_4^+ uptake under both NH_4^+ conditions.

4.4.5 OsAKT1 mediates K^+ influx under low Na^+ and NH_4^+ conditions

The response of AKT1 to the presence of Na^+, NH_4^+, and K^+ has been examined by several studies. These studies showed that the K^+ permeability of AKT1 was between 55-63% in the absence of NH_4^+, and with an external K^+ concentration of 10-1000 μM (Spalding et al., 1999). An earlier study showed that KAT1 and AKT1 perform a similar role in K^+ selectivity. This was 20-fold more than it was for Na^+ and NH_4^+ (Bertl et al., 1995). Similarly, the orthologous DmKT1 was shown to be a K^+ selective channel in potassium-based buffer only (Scherzer et al., 2015). However, the present study showed that the overexpressoion of *AKT1* resulted in a higher K^+ influx

than in the wild type when just 1 mM NaCl was added, and under a low NH_4^+ concentration condition (Fig 4.5a, b). This result confirms that OsAKT1 is a K^+ selective channel, and that it discriminates between K^+ and Na^+, always choosing Na^+ in the presence of a very low NH_4^+ concentration. This function of OsAKT1 was diminished when the root was exposed to 40 mM NaCl, and surprisingly, there was no significant difference between the genotypes (Fig 4.5c, d). There could be several explanations for this data. First, studies have shown that, in the root protoplast of rice plants, the transcriptional level of *AKT1* is down-regulated, in response to salt stress. Thereby, the inward K^+ currents are significantly reduced, prompting the suggestion that OsAKT1 represents a dominant salt sensitive K^+ uptake channel in rice (Fuchs et al., 2005; Golldack et al., 2003). Second, it was noted by another study that all genotypes showed K^+ efflux in response to severe salinity. This could be due to similarities in the electro-chemical features of potassium and sodium, where the latter can compete with K^+ on the binding sites in the plasma membrane both under low and high-affinity K^+ channels and transporters (Shabala and Pottosin, 2014). Na^+ uptake crosses the plasma membrane to cause significant membrane depolarization, which then causes K^+ efflux through the outward-rectifying, depolarisation-activated K^+ channel GORK (Shabala and Cuin, 2008). The toxic level of cytosolic Na^+ also leads to an increase in the cytosolic Ca^{2+} concentration at the same time, increases H_2O_2 production, which in turn activate both GORK (Demidchik et al., 2010) and NSCCs (Demidchik et al., 2003) causing further K^+ efflux. Therefore, in response to salinity, the reduction in the transcriptional level, together with high K^+ flux may have concealed the electrophysiological mechanism of OsAKT1, and so resulted in a non-significant response.

The interaction between K^+ and NH_4^+ has been studied by Wang et al. (1996) who showed that NH_4^+ inhibited K^+ flux under high Na^+/low K conditions. Later, Spalding et al. (1999) found that NH_4^+ specifically inhibited the non-AKT1 component by competing for K^+ binding sites on the transporters. The data of the present study showed that OsAKT1 function in the overexpressors

was inhibited when the external concentration of NH_4^+ increased from 20 μM to 2 mM, but with no significant difference being noted among all genotypes. This suggested that OsAKT1 is also sensitive to high levels of NH_4^+, and that NH_4^+ has an inhibitory effect not only on the non-AKT1 component, but also on OsAKT1. A previous study showed that rice is affected by NH_4^+ toxicity under a high-affinity K^+ transport system (Szczerba et al., 2008a) identical to the experimental condition used in the present study. Similarly, ten Hoopen et al. (2010) found that net fluxes of K^+ and NH_4^+ were negatively correlated, as there is direct competition between them during uptake. In conclusion, the data from the present study showed that OsAKT1 mediates K^+ uptake both under low Na^+ and NH_4^+ conditions (Fig 4.5a). Salt stress may have inhibited the physiological function of OsAKT1 by both down-regulating the *OsAKT1* transcripts and imposing K^+ efflux via GORK and NSSCs, which, in turn, could have concealed the function of OsAKT1. Finally, NH_4^+ had a negative effect on OsAKT1, thereby causing a reduction in the K^+ influx in the elongation zone of rice plants.

Chapter 5

Assessing the role of OsHKT1;5 in rice salt tolerance

5.1 Introduction

The exposure of plants to high levels of salt in the rhizosphere causes toxic levels of Na^+ and Cl^- ions to accumulate in cellular and extracellular compartments. This negatively affects the cells' metabolic activity, largely by affecting the acquisition and homeostasis of potassium, which are needed to activate a number of cytosolic enzymes and maintain membrane potential (Maathuis and Amtmann, 1999; Shabala and Cuin, 2008). Salt tolerant plants have developed many mechanisms for minimising the transport of Na^+, and accumulation of excessive levels of it in photosynthetic tissues. These mechanisms, known as salt exclusion mechanisms, include: a) selective uptake of ions by root cells, b) Na^+ exclusion from the root via the salt overly sensitive 1 (SOS1) protein encoding plasma membrane Na^+/H^+, antiporter, c) minimizing of xylem Na^+ loading, d) maximizing of xylem unloading via retrieval of Na^+ from xylem vessels (Munns and Tester, 2008) and e) control of phloem loading (Munns, 2002).

Glycophytic plants, such as rice, use these salt excluding mechanisms as their primary strategy for preventing toxic accumulation of salt in photosynthetic tissues (George et al., 2012). In such plants, maintenance of a relatively high K^+/Na^+ ratio, especially in shoots is a key factor in the development of salt tolerance (Hauser and Horie, 2010). The concerted activity of a large number of transporters located in plasma and vacuolar membranes plays an important role in determining the K^+/Na^+ ratio in plant cells, through Na^+, K^+ selective and non-selective pathways (Maathuis and Amtmann, 1999). The long-distance transport of Na^+ relies on xylem loading, which can be achieved by passive loading that is mediated by Na^+-permeable ion channels located at the xylem-parenchyma interface, and/or active loading, mediated by either SOS1 or a cation–Cl (CCC) co-transporter (Shabala, 2007). The *TrK/Ktr/HKT* family (Transporter of K^+/ K^+

transporter / High-affinity K^+ Transporter) of membrane transporters is assumed to be involved in various functions from K^+ or Na^+ uptake to maintenance of membrane potential, ion homeostasis, and Na^+ recirculation from shoot to root in plants (Corratgé-Faillie et al., 2010). The HKT family can be divided into two distinct sub-families based on their gene structure and whether they have a G or and S in the pore loop with some exceptions (Platten et al., 2006). The transporters in sub-family one, such as OsHKT1;1 and OsHKT1;5 are more selective to Na^+ over K^+, while those in sub-family two are selective to K^+ or alternatively, will transport both ions (Platten et al., 2006). Several studies have revealed that sub-family one has a detrimental effect on the maintenance of a high K^+/Na^+ ratio in plants (Byrt et al., 2007; Davenport et al., 2007). It has been reported that the Arabidopsis genome possesses only one gene *AtHKT1;1* encoding HKT transporter (Uozumi et al., 2000) while, in the rice genome, a total of nine *HKT* genes have been found, divided between sub-families 1 and 2 (Platten et al., 2006). When the gene *AtHKT1;1* was being expressed in the outer cells of the roots of rice plants, they exhibited a lower rate of root-to-shoot transport of Na^+, correlated with an up-regulation of *OsHKT1;5* expression. Eventually, these plants were found to have a lower shoot Na^+ content and to be more salt tolerant compared to the WT (Plett et al., 2010).

It has been shown that *HKT1;5*-like genes are candidates for *Nax2* in durum wheat, and for *Kna1* in bread wheat (Byrt et al., 2007). *Nax2* and *Kna1* have the same phenotype as *SKC1* that encodes OsHKT1;5 in rice (Ren et al., 2005), conferring salt tolerance by controlling sodium retrieval from the shoot, and increasing rates of K^+ transport from root to shoot via allowing other proteins in the plant to keep transporting K^+ to the shoot maintaining high K^+:Na^+ levels (James et al., 2006). Therefore, the majority of previous studies have focused on the direct physiological mechanism of *SKC1* locus (OsHKT1;5) on Na^+ transport from roots to shoots. However, a recent study showed that the *Nax* loci also regulate the activity and the expression level of SOS1-like Na^+/H^+ antiporter in the xylem tissue of wheat (Zhu et al., 2016). This suggests that *Nax* loci confer two complementary mechanisms: via retrieval of Na^+ from the xylem to the root stele via HKT1;5,

and via a reduction in the rate of Na^+ loading into the xylem by salt-overly-sensitive (SOS1) (Zhu et al., 2016). Thus, the question remains as to what extent the role played by *SKC1* locus in reducing the accumulation of Na^+ in the shoot can be attributed to HKT1;5 function, or it was predominantly due to the down-regulation of *HKT1;5*. In the plan terms: was HKT1;5 playing a primary or secondary (after SOS1) role in the reduction of Na^+ in the shoots?. Also, the HKT1;5 transporter is located in the plasma membrane of the xylem parenchyma (Zamani Babgohari et al., 2013), while *SOS1* expression was reported for both epidermal cells in the root apex and in the parenchyma cells in the xylem/symplast boundary of roots, stems, and leaves (Shi et al., 2002). Thus, if plants increase SOS1 activity in an attempt to eliminate Na^+ uptake by the root, this has the disadvantage of also concurrently increasing the rate of xylem Na^+ loading. Whether this can be prevented or whether it is possible that the HKT1;5 operation may modify expression levels and/or activity of SOS1 (or some other genes) involved in Na^+ transport and sequestration are questions that to the best of our knowledge, have not been investigated in the literature, to date.

In this study, the knocked-down *OsHKT1;5* genotype is compared with its wild type counterpart, in order to evaluate the physiological role of OsHKT1;5 in the adaptive responses of rice to salt and drought stresses, and to provide some insights into the regulation of its expression in various root tissues. The major finding of the present study is that OsHKT1;5 plays an indirect role in altering the functions of other genes.

5.2 Materials and Methods

5.2.1 Plant materials

Mature seeds of rice plants, *O. sativa L. Japonica* cv Dongjin wild type, and its mutant, OsHKT1;5 (4A-02764) were supplied by Dr Chang-deok Han, Rice Functional Genomics, National Institute of Agricultural Biotechnology, South Korea. The expression profile of OsHKT1;5, both in the mutant and its wild type, is fully explained in the supporting information section.

5.2.2 The phenotyping experiment

The growth experiment was carried out in the glasshouse and the parameters were measured as the methods described in the first experimental chapter.

5.2.2.1 Microelectrode preparation

The microelectrode preparation was carried out using a method developed by (Shabala et al., 2006a) and (Shabala et al., 2013), as described in the first experimental chapter. The electrode tip was quickly front-filled with the corresponding LIX which listed in the following table (Table 5.1).

Table 5. 1 Ionophores (LIX) and the back-filling solutions which were used to prepare the microelectrode of the selected ion.

Ion	Ionophore (LIX)	Back-filling solution (mM)
Na^+	N,N′,N″-Triheptyl-N,N′,N″-trimethyl-4,4′,4″-propylidynetris(3-oxabutyramide)	500 NaCl
K^+	Valinomycin	500 KCl
Ca^{2+}	(-)-(R,R)-N,N′-(Bis(11-ethoxycarbonyl)undecyl)-N,N′-4,5-tetramethyl-3,6- dioxaoctanediamide	500 $CaCl_2$
H^+	4-Nonadecylpyridine	15 NaCl+ 40 KH_2PO_4, (pH 6)

To calibrate the microelectrodes, an appropriate set of three standard solutions, (Table 5.2) covering the expected range of targeted ion, was prepared. The procedure of calibration is described in the first experimental chapter.

Table 5. 2 Measured ions and their standard calibration solutions

Ion	Standard calibration solutions
Na^+	10, 50, 100 mM NaCl
Ca^{2+}	100, 200, 400 µM $CaCl_2$
K^+	250, 500, 1000 µM KCl
H^+	Buffers with pH 5.30, 6.67, 7.65

5.2.2.2 Experimental protocol

Root preparation was carried out as described in the first experimental chapter. The distilled water was used as a growth medium for seed germination. The BSM bathing medium consisted of 200 µM NaCl, 100 µM $CaCl_2$, and 200 µM KCl. The pH level of the BSM solution

was maintained at around 5.4-5.5. For conditioning, roots were left in the bathing solution for approximately 30-60 min.

The measurement was carried out as the procedure was described in the first experimental chapter. The treatments of 80 mM KCl, 1 mM copper ascorbate (Cu/A) the hydroxyl radical ($OH^{\bullet}$)-generating mix, and 10 mM H_2O_2 were used to achieve the aim of this study. The ion flux was measured in the mature and elongation zones and the stele of rice root.

Root stellar tissues isolation was carried out as the procedure was described by (Shabala et al., 2010). The rice roots were immersed in a BSM solution to avoid root dryness, then root segments (5 cm in length) were cut from the mature zone of rice roots. The stellar tissues were gently extracted by slicing one of the root segment end and pulling the cortical tissue using two fine tweezers under binocular microscope. The isolated stellar segments were used for MIFE experiment as described in the first experimental chapter. Measurements started 3–4 h later to avoid any potential confounding effects of mechanical damage during segment isolation. The steady fluxes were measured for 5–10 min to make sure that a steady-state condition was reached. Then salinity treatment was given, and transient Na^+, K^+ and H^+ kinetics were measured for another 30 min

5.3 Results

5.3.1.1 Knock-down of *OsHKT1;5* expression in the root impaired growth of the mutant rice plants under salt stress

The growth and biomass accumulation in both the WT and KD lines were significantly affected by salinity stress, but to differing degrees: under 40 and 80 mM of NaCl the KD line showed a dramatic growth reduction of 25% and 72%, respectively, whereas the WT showed only a slight growth reduction of 5% and 25% respectively, compared to the control. The number of tillers per plant (Fig 5.2.b), the WT showed no significant difference, while in the KD line the number of tillers decreased significantly ($p \leq 0.01$) by 41% when the salt concentration was increased to 80 mM NaCl in the hydroponic medium (Fig 5.2.b).

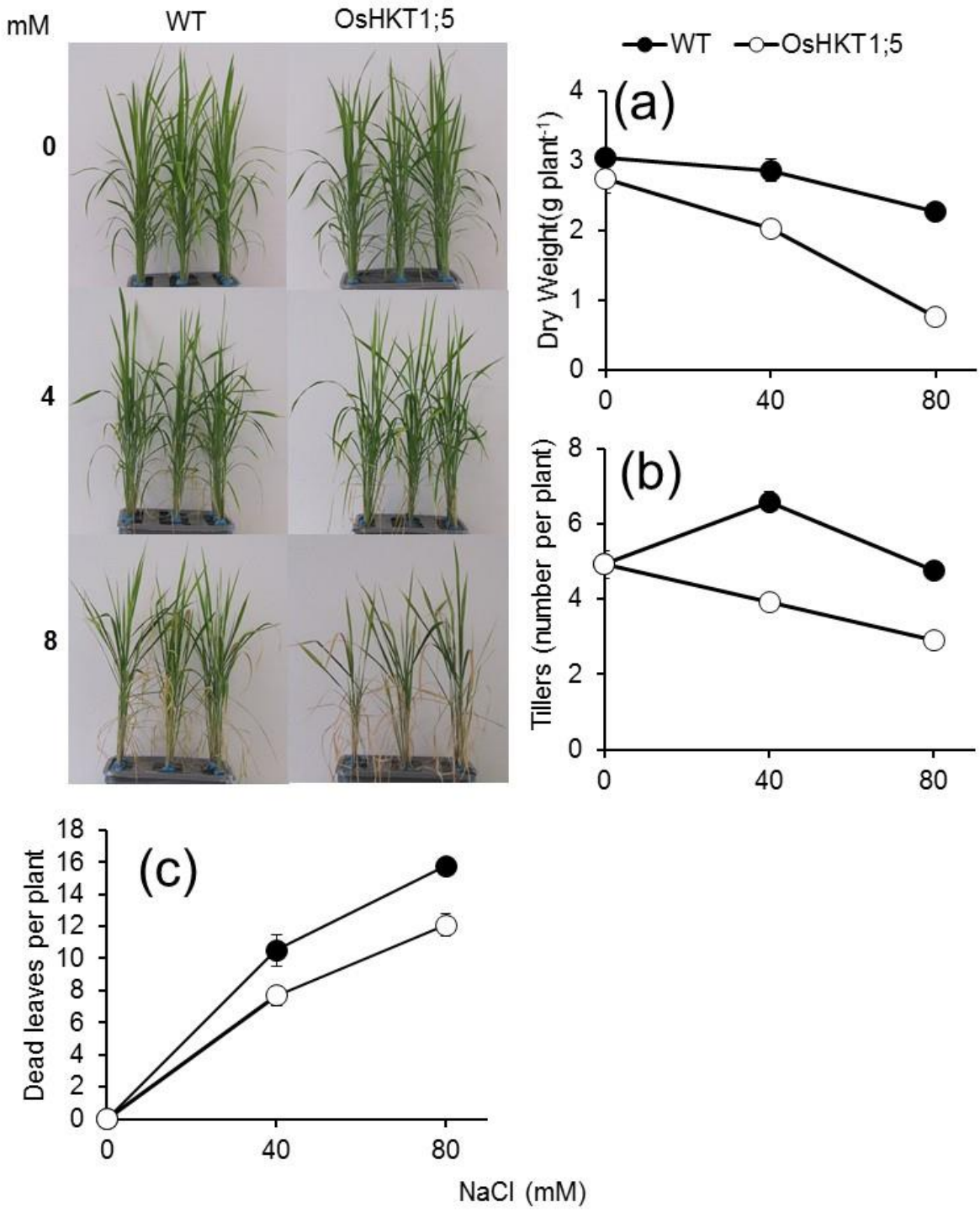

Figure 5. 1 Salinity significantly impaired growth of rice plants. The KD line had (a) lower dry weight, (b) fewer number of tillers per plant, (c) fewer dead leaves per plant. Both genotypes were grown under control conditions: 40, and 80 mM NaCl, respectively in a hydroponic system for 21 days. WT (closed circles), KD line (open circles). Data are the mean ± SE (n=18). No dead leaves were recorded for either genotype under control conditions (Fig 5.2.c). However, upon exposure of the plants to 80 mM NaCl the number of dead leaves was significantly ($p \leq 0.01$) higher: 16- and 12-fold increases compared to the control in the WT and KD lines, respectively (Fig 5.2.c).

The fact that WT plants exhibited strong vegetative growth and produced more tillers implying that the number of dead leaves was significantly higher in the WT compared to KD line.

Osmolality data was in line with the Na^+ content in the shoots of both genotypes (Fig 5.3.a). Plant's exposure to 80 mM NaCl significantly increased ($p \leq 0.01$) the shoot osmolality by 67%, and 213%, in the WT and KD line, respectively. Additionally there was a significant difference in osmolality between the wild type and the KD line under saline condition. (Fig 5.3.a).

Stomatal conductance (Gs) in the KD line was 17% higher than in the WT, under control conditions (Fig 5.3.b). The data recorded shows that the stomatal conductance was negatively correlated to the salt stress, as it dramatically decreased ($p \leq 0.05$) by 76%, and 82%, respectively in the WT and KD lines, when salinity was increased to 80 mM NaCl (Fig 5.3.a).

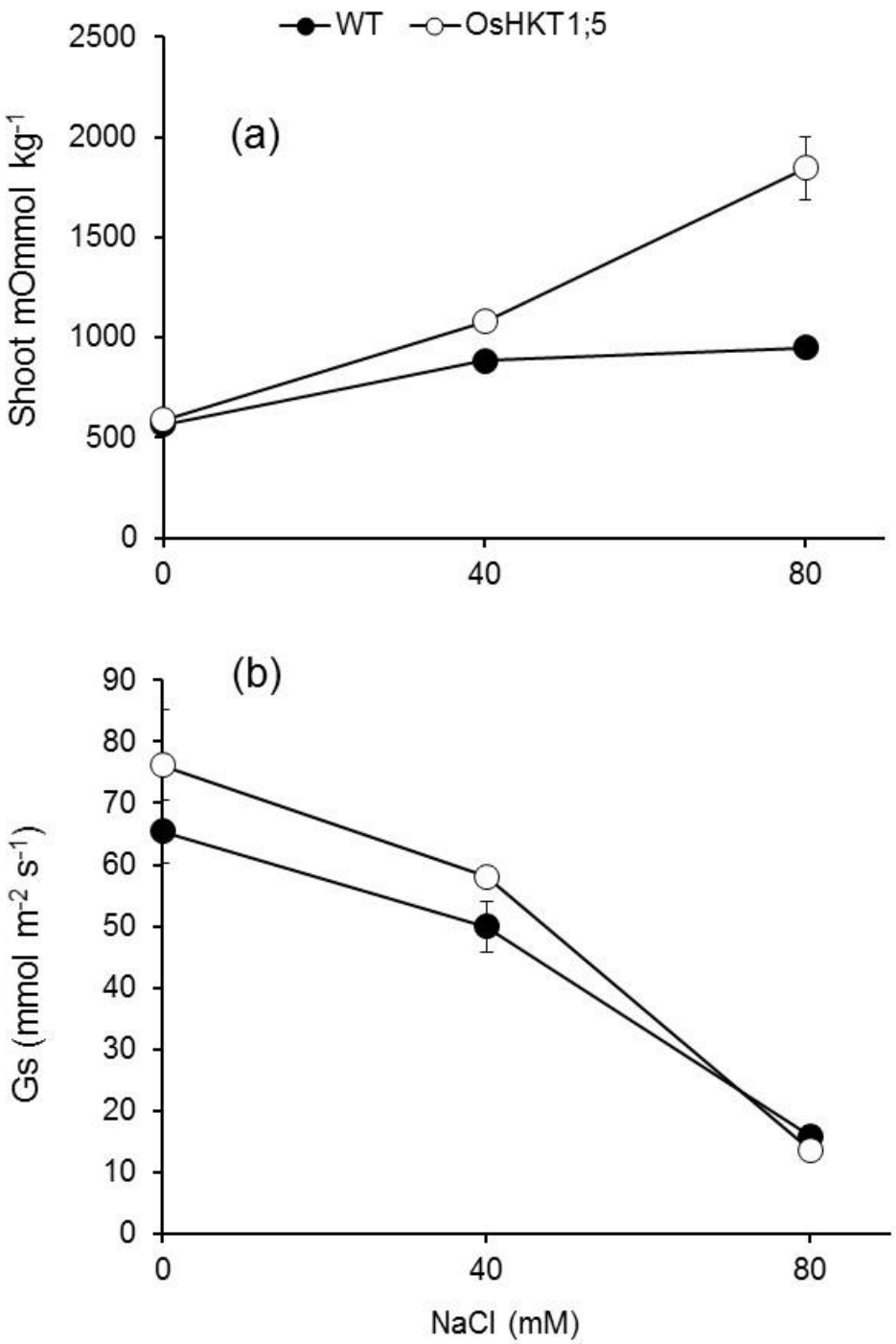

Figure 5. 2 Shoot osmolality (a), and stomatal conductance (b) in both the KD line and the WT. Both genotypes were grown under control conditions: 40, and 80 mM NaCl, respectively in a hydroponic system for 21 days. WT (closed circles), KD line (open circles). Data are the mean ± SE (n=18).

5.3.1.2 Knock-down of *OsHKT1;5* expression leads to increase in Na^+ shoot concentration of the mutant plants under salt stress

Both genotypes had a similar shoot and root Na^+ content under control conditions (Fig 5.4.a). However, under salt stress, the KD line showed a shoot Na^+ concentration that was eight times higher (Fig 5.4.a), and a root Na^+ concentration that was 13% lower, than the WT at 80 mM NaCl. In the WT, under salt stress conditions, the shoot K^+ concentration remained relatively constant, similar to that in the control plants. However, the K^+ content of shoots in the KD line showed a 4-fold ($p \leq 0.01$) decrease at 80 mM NaCl compared to the control (Fig 5.4.c).

The root K^+ content, a significant difference ($p \leq 0.05$) was observed between the genotypes under control conditions (Fig 5.4.d). This difference was even more marked under salt stress, with the root K^+ content decreasing by 71% and 52% respectively for WT, and KD line, at 80 mM NaCl compared to the control (Fig 5.4.d).

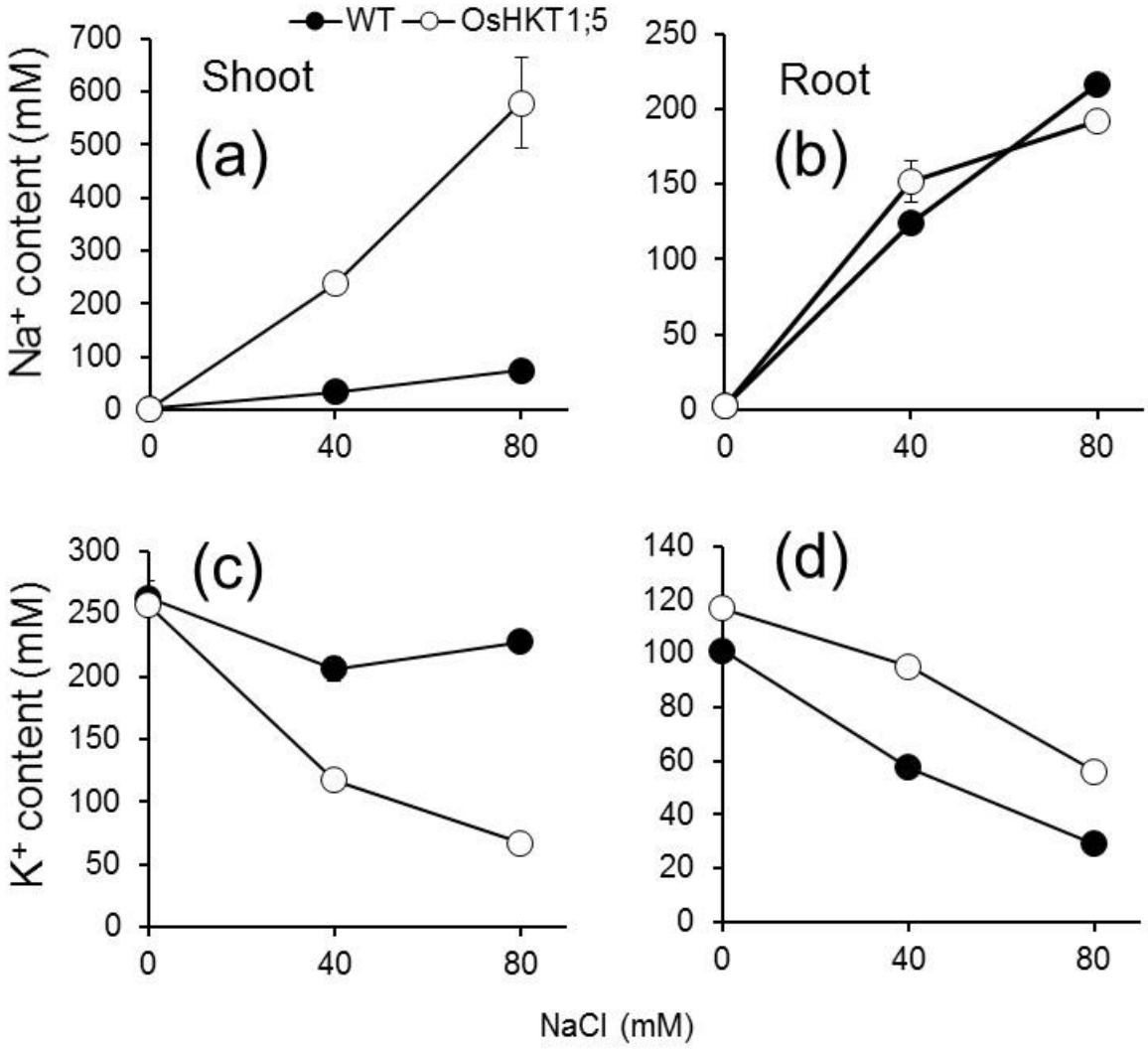

Figure 5. 3 The KD line had (a) a higher shoot Na^+ content. Indeed, (c) a lower shoot K^+ content in KD line caused a reduction in the K^+:Na^+ ratio. Root Na^+ content was higher in WT, while (d) under saline conditions, the root K^+ content was higher in the KD line than in the WT and Both genotypes were grown under control, 40, and 80 mM NaCl in a hydroponic system for 21 days. WT (closed circles), KD line (open circles). Data are the mean ± SE (n=6).

Salt stress significantly altered the K^+: Na^+ ratio both in shoots and roots of rice plants. Both genotypes had a similar K^+: Na^+ ratio in both shoots and roots under control conditions. Interestingly, when the salt concentration was increased to 80 mM NaCl, the WT showed significantly a higher K^+: Na^+ ratio in the shoots (calculated based on K^+ and Na^+ concentration data) compared to the KD line, but the opposite was true for the roots.

5.3.1.3 The knock-down of *OsHKT1;5* expression in the root of the mutant plants does not affect plant growth under drought stress

The KD line had a significantly ($p \leq 0.05$) lower dry weight compared to WT, both under control and drought stress conditions (Fig 5.5.a). Both genotypes exhibited the same trend when drought stress was applied, with the dry weights significantly decreasing by 37%, and 39% for WT and KD lines, respectively (Fig 5.5.a). SPAD values increased slightly, but not significantly ($p < 0.05$), in both genotypes when drought stress was applied, with no significant difference observed between the genotypes (Fig 5.5.b). Under drought stress, shoot osmolality ($p \leq 0.01$) and K^+ content ($p \leq 0.05$), increased significantly by 45% and 15% respectively in both genotypes (Fig 5.6.a.c).

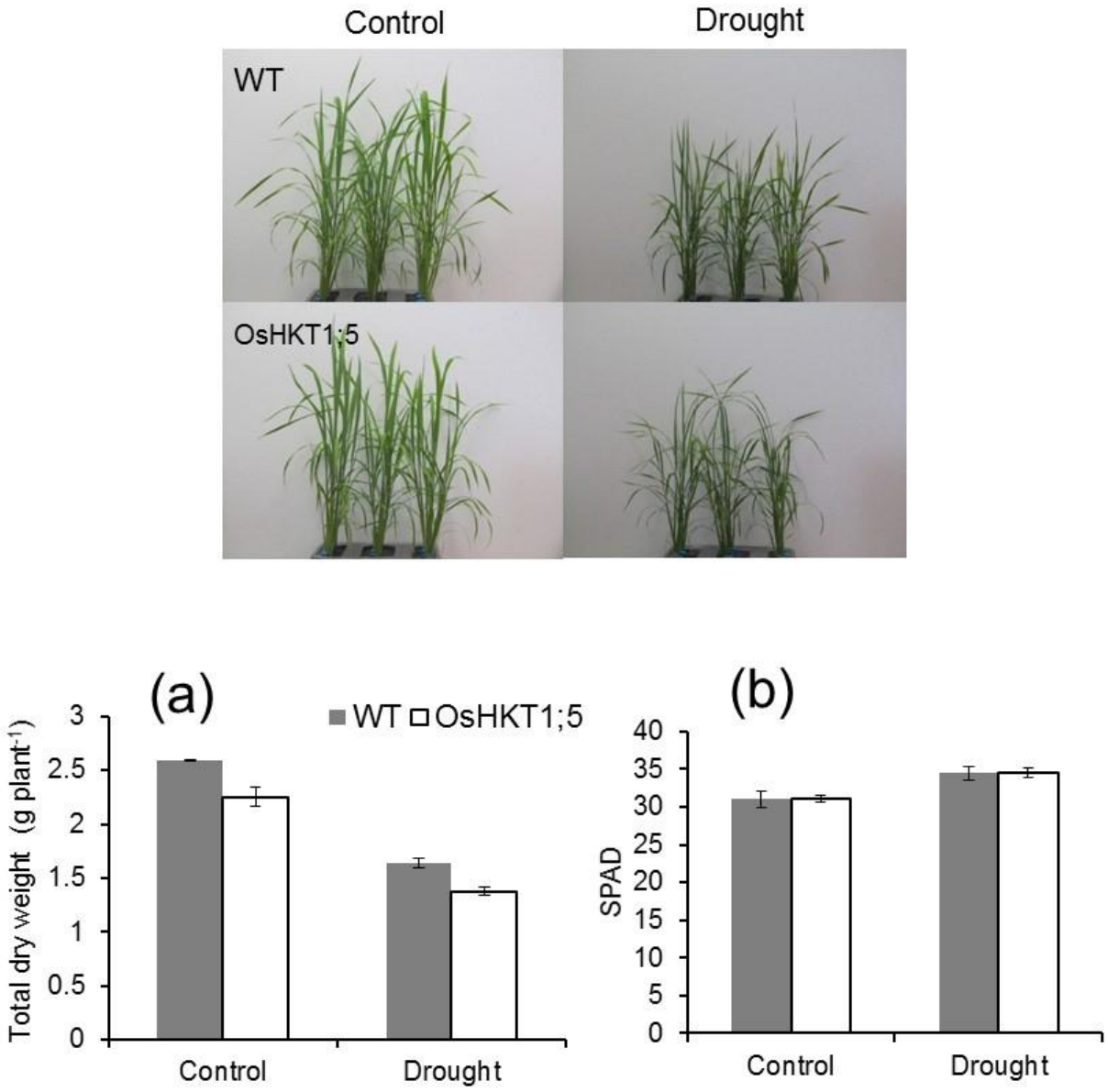

Figure 5. 4 Drought stress caused (a) a reduction in (a) dry weight while (b) the SPAD value increased for both genotypes. There was no specific correlation between *OsHKT1;5* expression and drought stress. Both genotypes were grown under control, and under drought stress caused by 15% of PEG (4000) for 21 days. Data are the mean ± SE (n=18).

No significant difference was recorded in the osmolality and Na^+, and K^+ content between the genotypes, under both control and drought conditions (Fig 5.6.a.b.c). Interestingly, both genotypes had a similar root Na^+ content, under control conditions, while the KD showed a 27% lower root Na^+ content, under drought stress conditions (Fig 5.6.d).

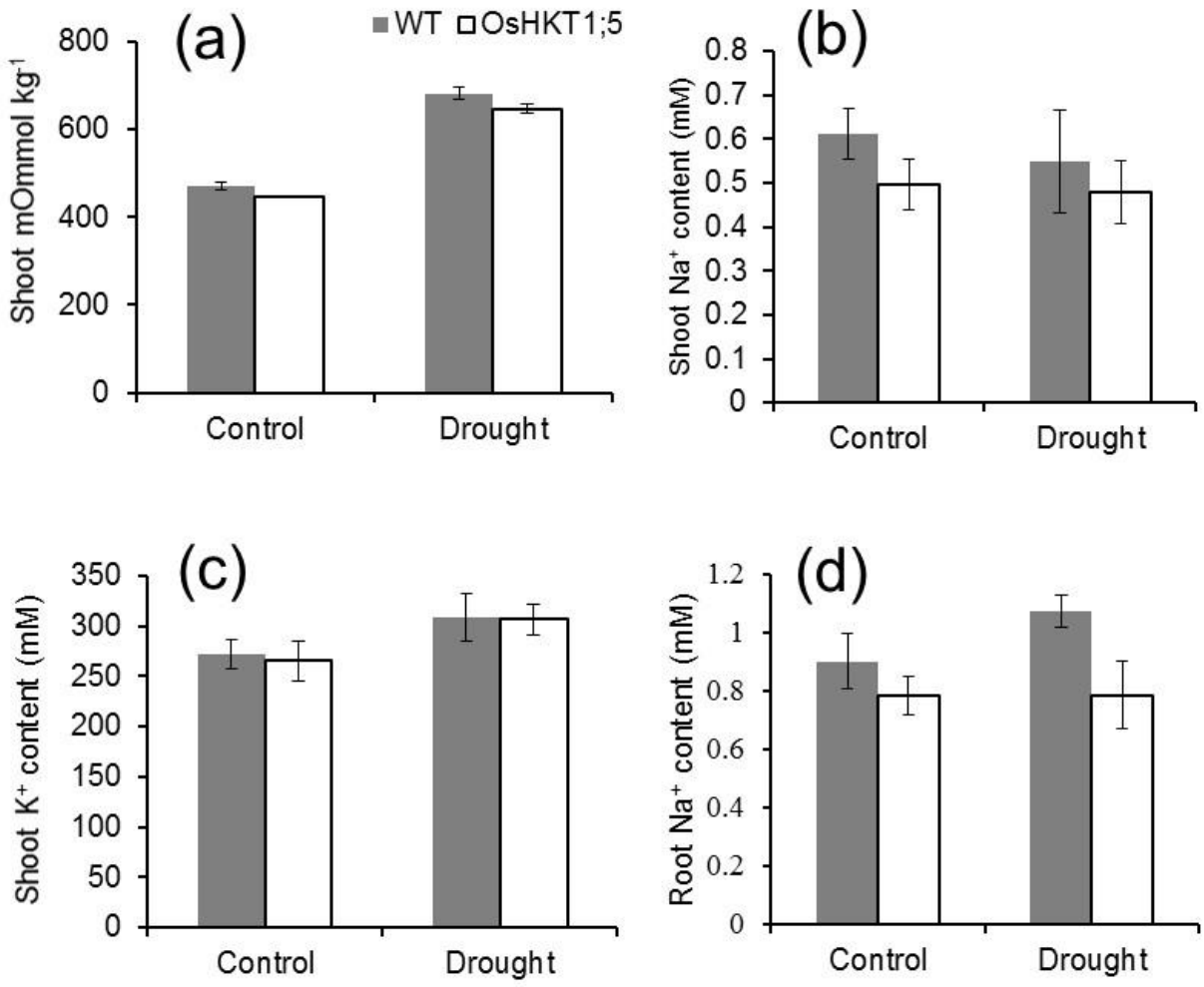

Figure 5. 5 (a) Shoot osmolality and (c) shoot K^+ content (b) shoot Na^+ content and (d) root Na^+ content of both genotypes under drought stress condition. The KD and WT lines were grown at control, and drought stress was applied using 15 % (w/v) of PEG (4000) for 21 days. Data are the mean ± SE (n=6).

5.3.2 Short term of salt exposure

5.3.2.1 The KD line had lower Na^+ influx in the elongation zone

The kinetic flux of ions in the epidermal cells of root was studied using non-invasive ion flux measuring MIFE technique. The addition of 80 mM of NaCl caused an immediate and marked influx of Na^+ into the epidermal cells of the root of both genotypes (Fig 5.7.a.b.c). Epidermal cells in the elongation zone of the KD line had a 6-fold lower Na^+ influx than WT under salt stress

lasting a maximum of 20 minutes (significant at $p \leq 0.01$; Fig 5.7.a). No significant difference (at $p < 0.05$) was found, between the genotypes in the mature zone (Fig 5.7.b). The difference between the two was also insignificant for Na^+ influx in the stele (Fig 5.7.c). The biggest Na^+ influx was in the epidermal cells of the elongation zone (Fig 5.7.a), followed by those in the stele (Fig 5.7.c) and then, those in the mature root epidermis (Fig 5.7.b).

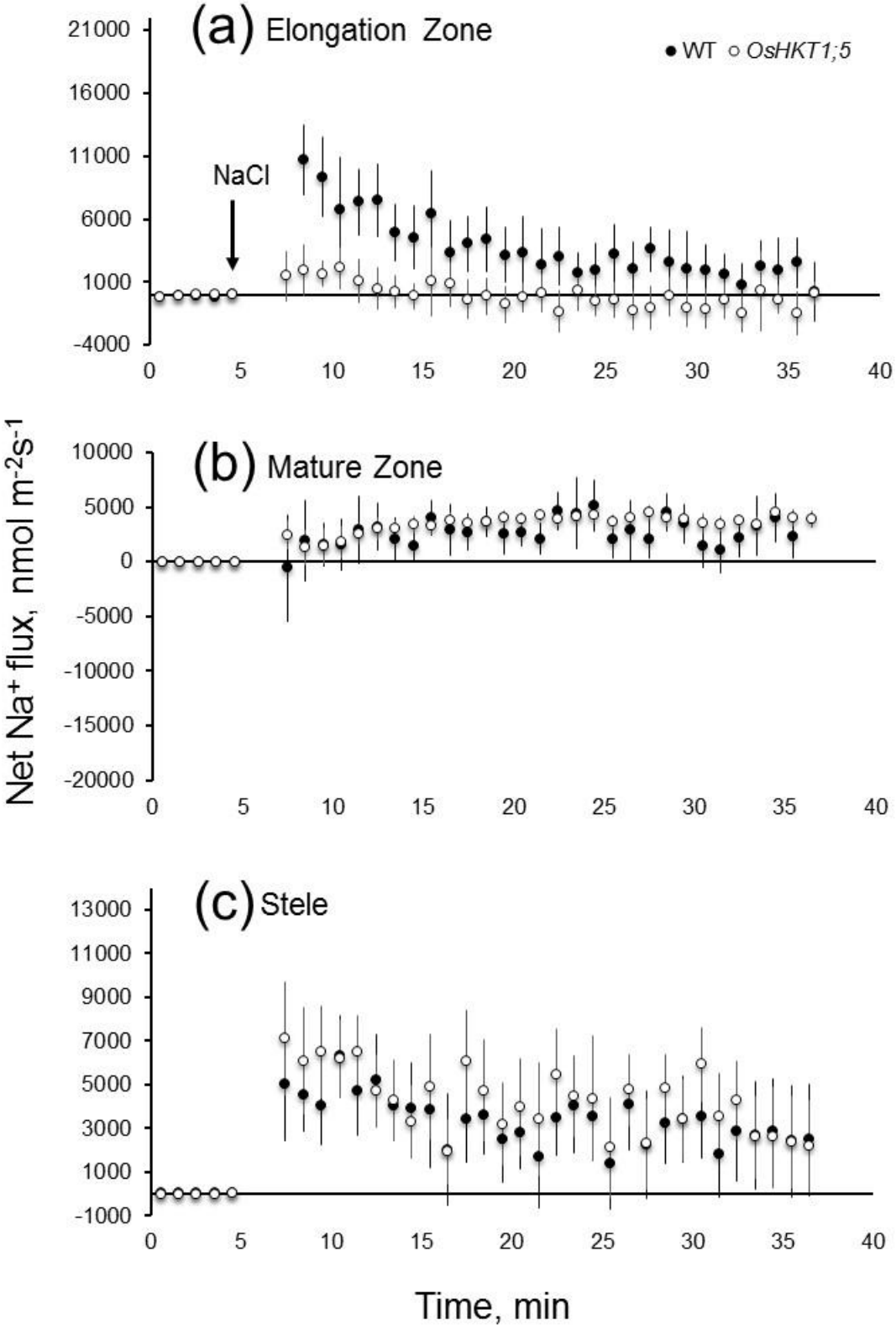

Figure 5. 6 Salt-induced Na^+ influx into (a) elongation zone, (b) mature zone, and (c) stele both of KD and WT lines of rice plants. Five-to-six-day-old seedlings of both genotypes were treated with 80 mM NaCl. Data are the mean ± SE (n=6).

5.3.2.2 Salt-induced K^+ efflux from the root epidermal cells of KD line is lower than that of the wild type

Exposure of roots to 80 mM NaCl resulted in considerable leakage of K^+ from the epidermal cells of the mature and elongation zones and stelar tissue in both genotypes (Fig 5.8.a.b.c). The responses differed, quantitatively, among the various parts of root tissue. The K^+ efflux from the elongation zone was 10-fold, and 3-fold higher than from the mature zone and stele, respectively (significant at $p \leq 0.05$; Fig 5.8.a). Furthermore, when stress was imposed, a kinetic study of K^+ flux showed that K^+ leakage was significantly ($p \leq 0.05$) lower in the elongation zone of the KD line than in that of the WT line (Fig 5.8.a), and relatively lower in the mature zones of both genotypes (Fig 5.8.b) and stele (Fig 5.8.c. Overall, in the root epidermal cells the KD line experienced lower K^+ efflux compared to the WT.

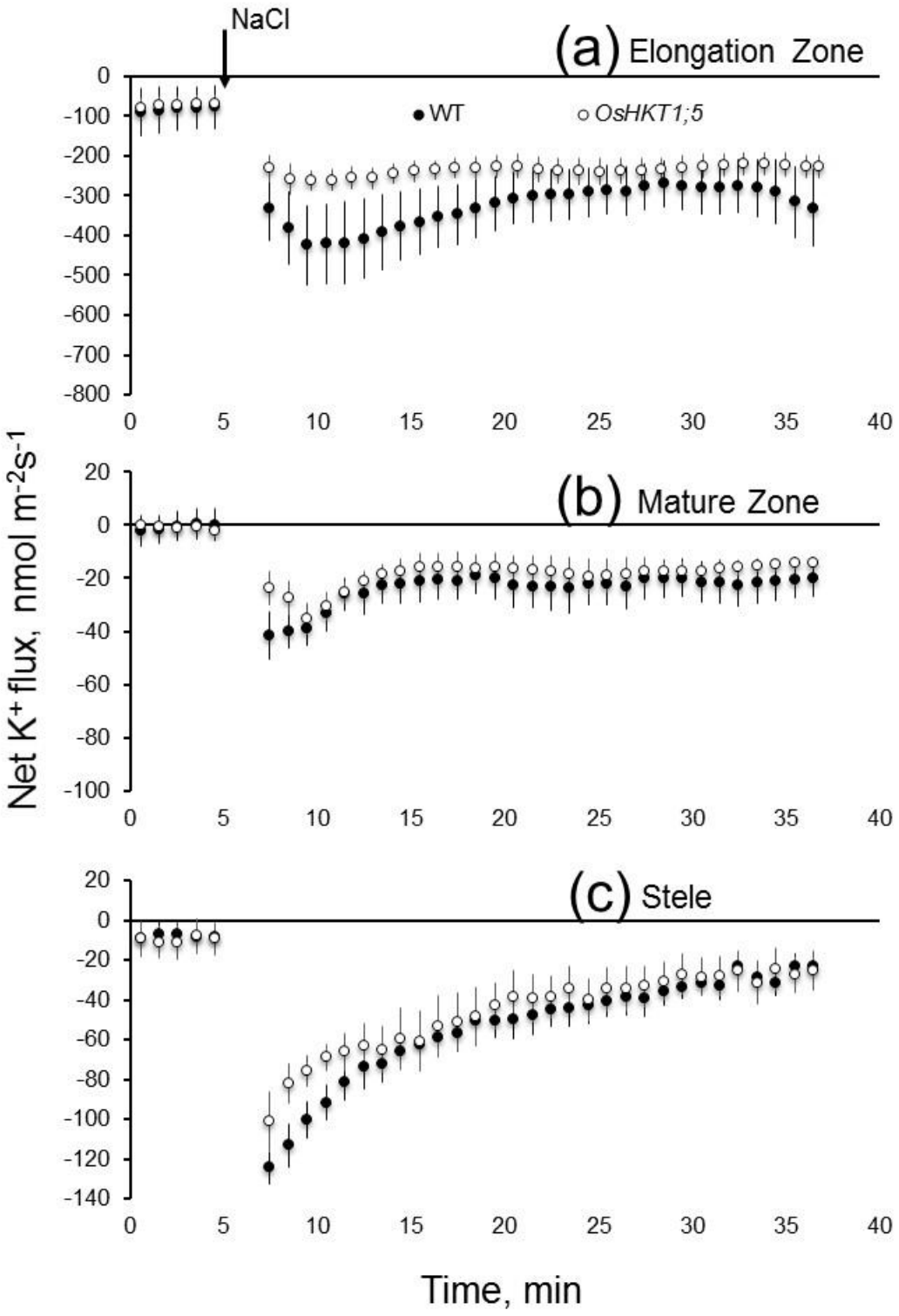

Figure 5. 7 Salt-induced K^+ efflux from (a) elongation zone, (b) mature zone, and (c) stele both of KD and WT lines of rice plants. The insets depict average Na^+ flux per minute. Five-to-six-day-old seedlings of both genotypes were treated with 80 mM NaCl. Data are the mean ± SE (n=6).

5.3.2.3 Salt treatment activates H^+ and Ca^{2+} efflux

Over the time of the study, salt stress induced an immediate H^+ efflux in the KD line, while H^+ flux gradually changed to efflux in the elongation zone of the WT (Fig 5.9.a). The two genotypes differed in their response to salt stress, with the KD line showing a significantly ($p \leq 0.01$) higher H^+ efflux compared to the WT, in both the elongation (Fig 5.9.a) and mature zones (Fig 5.9.b). The highest H^+ efflux was observed in the elongation zone (Fig 5.9.a) and the lowest in the stele (Fig 5.9.b). However, there was no significant difference between the genotypes, in terms of the H^+ efflux found in the stele (Fig 5.9.a).

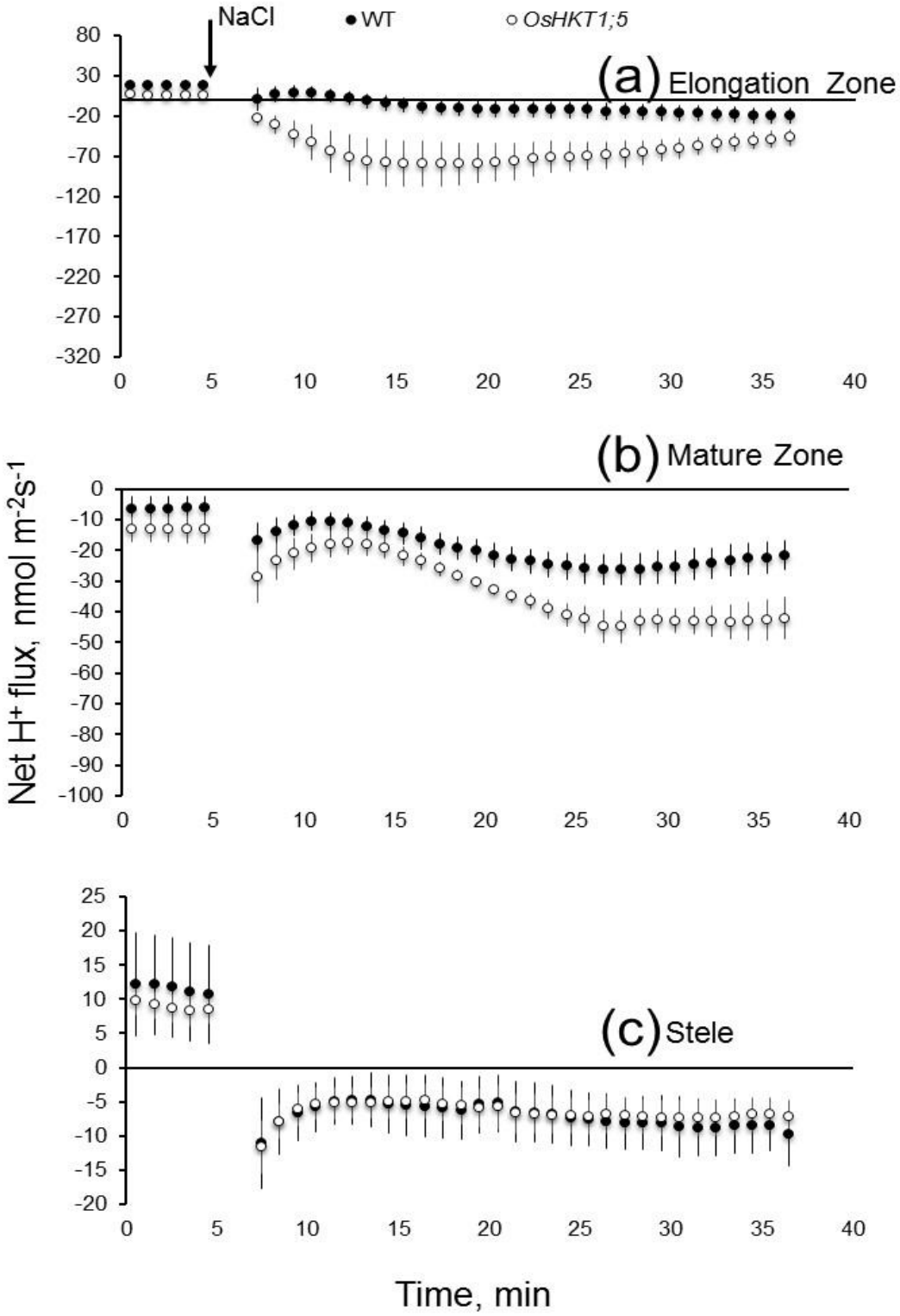

Figure 5. 8 Salt-induced H^+ efflux from (a) elongation zone, (b) mature zone, and (c) stele both of KD and WT lines of rice plants. The insets depict average H^+ flux per minute. Five-to-six-day-old seedlings of both genotypes were treated by 80 mM NaCl. Data are the mean ± SE (n=6).

The Ca^{2+} flux displayed a similar trend to the H^+ flux. Salinity (80 mM NaCl) caused a significant ($p \leq 0.05$) Ca^{2+} efflux from the epidermal cells (Fig 5.10). This salt-induced Ca^{2+} efflux

was significantly higher in the KD line compared to the WT both in the elongation (Fig 5.10.a) and mature zones (Fig 5.10.b).

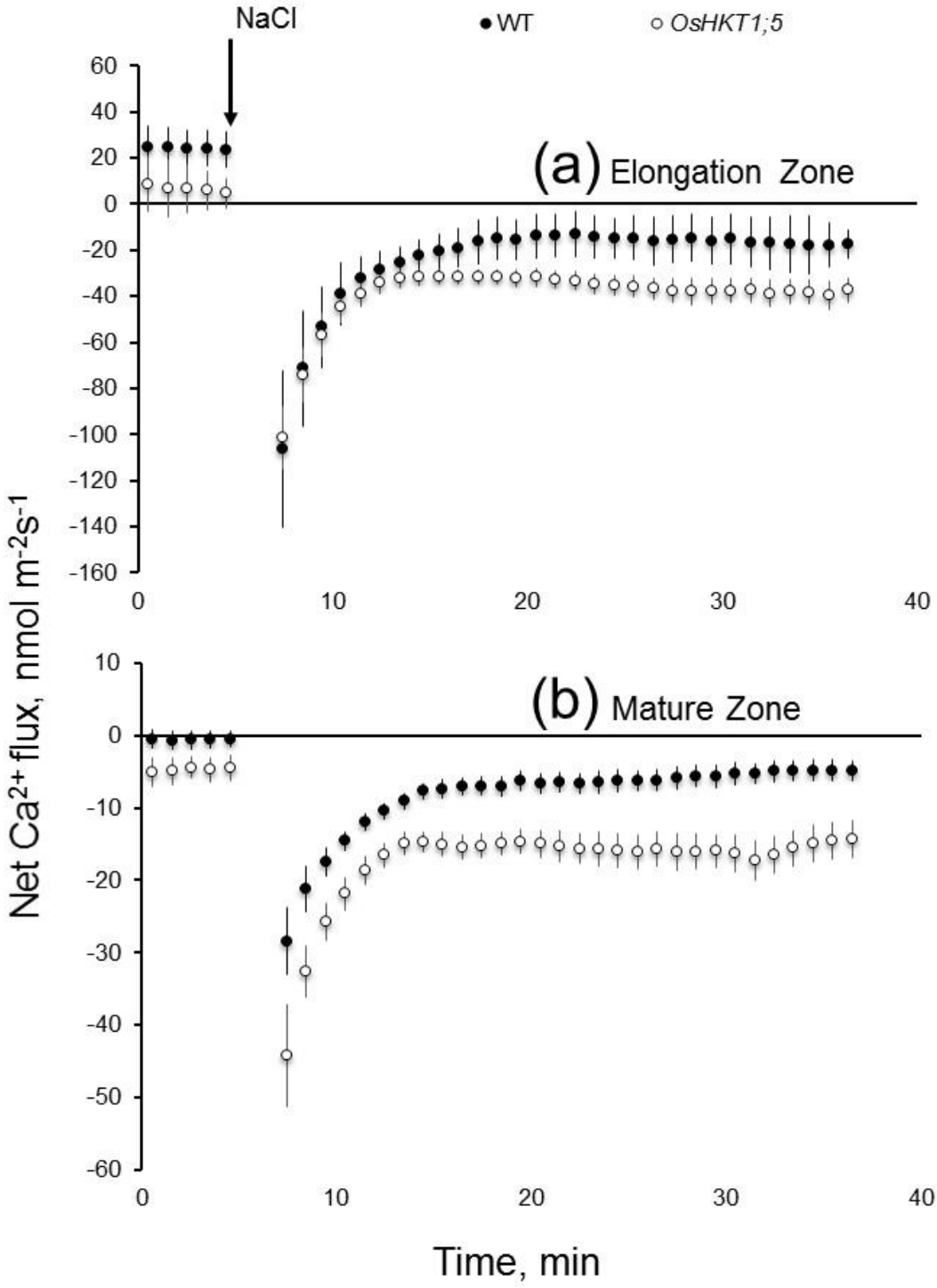

Figure 5. 9 Salt-induced Ca^{2+} efflux from (a) elongation zone, and (b) mature zone both of KD and WT lines of rice plants. The insets depict average Ca^{2+} flux per minute. Five-to-six-day-old seedlings of both genotypes were treated by 80 mM NaCl. Data are the mean ± SE (n=6).

5.3.3 Oxidative stress

5.3.3.1 ROS-induced ion fluxes from the roots of both WT and KD

Exposing roots of both genotypes to 1 mM copper ascorbate (Cu/A; a hydroxyl radical ($OH^{\bullet}$)-generating mix; (Demidchik et al., 2003) induced significantly high amounts of K^+ efflux in the elongation zone (Fig 5.11.a); however, these readings did not differ significantly (at $p < 0.05$) between the genotypes. The hydroxyl radical-induced K^+ efflux was immediate, and increased gradually over time, reaching a peak value after 3 min in both genotypes (Fig 5.11.a). Then, it gradually recovered, but remained higher than the K^+ flux under control conditions, until the end of the measurement period. Oxidative stress, imposed by adding 10 mM H_2O_2, also triggered a pronounced K^+ efflux (Fig 5.11.a), although not as strong as when the hydroxyl radical ($OH^{\bullet}$) was added. Also, no significant ($p \leq 0.05$) difference between the genotypes was found. The hydroxyl radical ($OH^{\bullet}$) induced an initial Ca^{2+} efflux followed by a Ca^{2+} influx, reaching a peak in the elongation zone after 12 min (Ca^{2+} influx $\approx$ 116 nmol $m^{-2}s^{-1}$) (Fig 5.11.b).

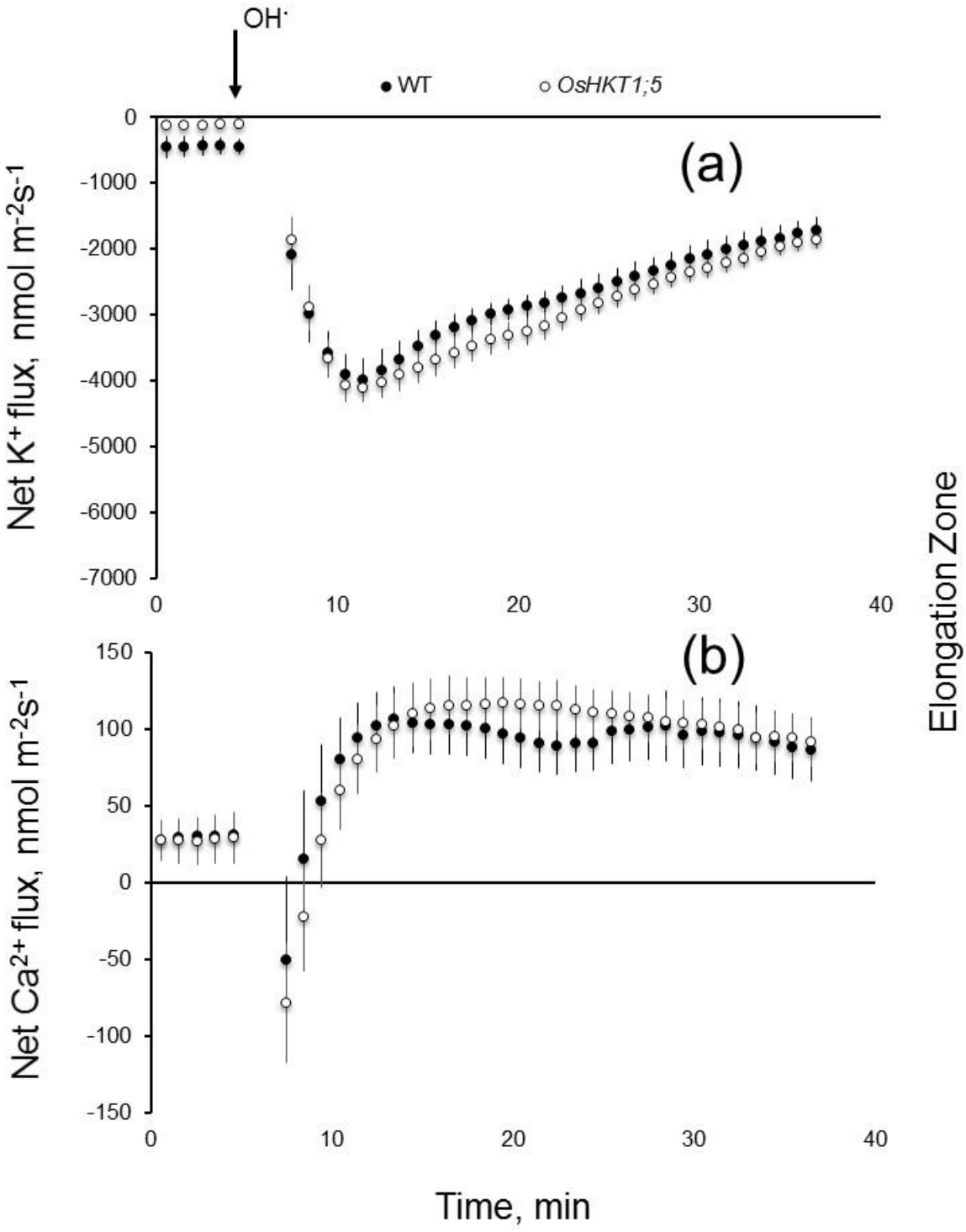

Figure 5. 10 OH•-induced (a) K^+ efflux and (b) Ca^{2+} influx in the elongation zone of KD and WT lines of rice plants. The insets depict average flux values per minute. Five-to-six-day-old seedlings of both genotypes were treated with 1 mM copper-ascorbate mixture. Data are the mean ± SE (n=6).

Again, no significant difference was found between the genotypes. H_2O_2 induced an immediate Ca^{2+} influx in the KD line, and a gradual Ca^{2+} influx in the WT. Although both

genotypes demonstrated a significant response to H_2O_2, the Ca^{2+} influx was significantly ($p \leq 0.01$) higher in the KD line than in the WT (Fig 5.12.b).

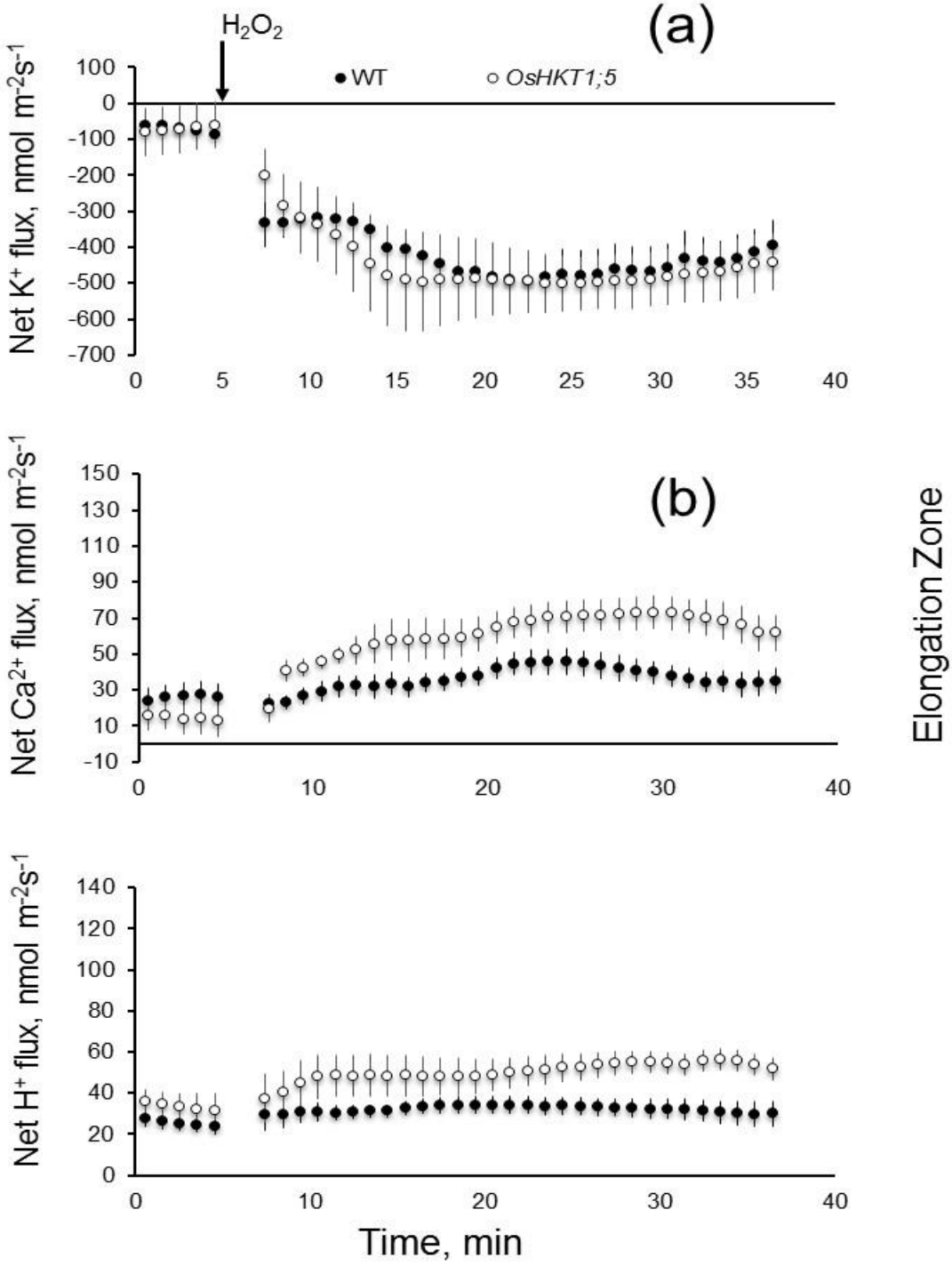

Figure 5. 11 H_2O_2-induced (a) K^+ efflux and (b) Ca^{2+} influx in the elongation zone of KD and WT lines of rice plants. The insets depict average flux values per minute. Five-to-six-day-old seedlings of both genotypes were treated with 10 mM of hydrogen peroxide (H_2O_2). Data are the mean ± SE (n=6).

5.4 Discussion

5.4.1 Knock-down of *OsHKT1;5* in the root of the mutant plants results in high levels of Na^+ in photosynthetic tissues

For glycophyte plants, the ability to maintain a low Na^+ content in the shoots, along with a high K^+/Na^+ ratio throughout the entire tissue of the plant, is vital for coping with drought conditions. There are three major mechanisms that are reported to minimise Na^+ accumulation in shoots while maintaining a high cytosolic K^+/ Na^+ ratio in rice plants: (1) Na^+ extrusion from the root, (2) Na^+ sequestration in vacuoles, and (3) Na^+ unloading from the xylem vessel (Horie et al., 2012). The HKT family of transporters is also considered to play an important role in maintaining low Na^+ levels in shoot tissue (Almeida et al., 2013; Platten et al., 2006). Also, it has been suggested that the OsHKT1;5 transporter is important both for the retrieval of Na^+ from xylem tissue and the prevention of further Na^+ accumulation in shoots (Cotsaftis et al., 2012). The results of ion content analysis of the KD and WT lines supports the notion that the KD of *OsHKT1;5* resulted in a high shoot Na^+ content and an accompanying disturbed K^+/Na^+ ratio, under varying salt conditions. Na^+ shoot content was 335-fold higher in the KD line compared to the control, while the WT succeeded in reducing the shoot Na^+ content, because it has a normal *OsHKT1;5* expression. Furthermore, due to the similar electro-physiological features of Na^+ and K^+, any increase in the concentration of Na^+ in the growth medium would necessarily affect the influx of K^+ into plant tissues, eventually changing the K^+ content of the shoots. Data obtained during this study showed that the shoot K^+ content of the KD line genotype was significantly lower than that of the WT line, which remained relatively constant (Fig 5.4.c). Thus, in the KD line, the shoot K^+ content negatively correlates to the shoot Na^+ content. Consequently, knock-down of *OsHKT1;5* expression in the root indirectly influenced the K^+:Na^+ ratio that is a crucial for plant growth. As a result, the K^+: Na^+ ratio was significantly lower in the KD line than in the WT under varying salt conditions. As a conclusion, It has been reported in wheat that Na^+ unloading from the xylem of

roots predominantly is mediated by *TmHKT1,5-A* gene, while *TmHKT1;4-A2* is mediated the Na^+ excluding from the leaf sheaths (Byrt et al., 2007; Huang et al., 2006; James et al., 2006) Knocking- down of *OsHKT1;5* expression on the root of the mutant plants lead to impair it growth as compared to the wild type under the salinity condition. Although, the mutant showed higher *OsHKT1;5* expression in the bleads and sheaths, that did not help the mutant plants to survive under salinity condition..

5.4.2 Knock-down of *OsHKT1;5* may increase the functional expression of *OsSOS1* in roots

It has been reported that salt stress induces an increase in H^+-ATPase activity, and that there is a positive correlation between salt tolerance and H^+-ATPase activity in different plant species, such as Arabidopsis (Ayala et al., 1996; Bose et al., 2013), barley (Adem et al., 2014), and the broad bean (Shabala, 2000). In view of these findings, the MIFE technique was thought to be a suitable proxy to study H^+-coupled ion exchange in plasma membrane (PM). On the one hand, an Na^+/H^+ exchanger encoded by the *SOS1* gene that is fueled by H^+-ATPase is an example of this coupling (Sun et al., 2009). On other hand, increasing the H^+-ATPase activity may provide a further driving force for potassium uptake via voltage-gated channels, or for high affinity K^+ uptake via HAK/KUP transporters (Shabala and Pottosin, 2014). In accordance with these predictions, H^+ data for the KD line revealed a significant increase in H^+ efflux, both in elongation and mature zones, upon exposure to 80 mM NaCl (Fig 5.9). However, when the KD and WT lines were compared in terms of the H^+ efflux from stele, no significant difference was found. These findings suggest that knock-down of *OsHKT1;5* up-regulated the activity PM H^+-ATPase, leading to a greater pH gradient across the plasma membrane in the epidermal cells of rice roots. This gradient may then provide the driving force for a plasma membrane SOS1 Na^+/H^+ exchanger to move Na^+ from the cytoplasm to the apoplast via SOS1 (Ayala et al., 1996), thus providing an explanation for the observation of higher Na^+ efflux in the KD line compared to the WT. These

observations are consistent with the finding of an earlier study on wheat by (Zhu et al., 2016), in which the *Nax2* (orthologe of OsHKT1;5) locus was also found to have affected the SOS1-like Na^+/H^+ exchanger activity in the epidermis. As a result of this, a higher Na^+ efflux was observed in the elongation zone of the KD line because of preferential expression of the SOS1 transporter in the epidermal cells (Shi et al., 2002). However, there was no significant difference between the genotypes, with respect to Na^+ flux in the mature zone. Hence, the knock-down of *OsHKT1;5* may increase the functional expression of *SOS1*, resulting in effective Na^+ exclusion from the elongation zone of root, as shown by the data. Given that the elongation zone represents a very small portion of the root, increasing the SOS1 activity at the epidermis would not be sufficient to completely prevent Na^+ reaching the xylem tissue. At the same time, knock-down of the *OsHKT1;5* expression in the xylem parenchyma is responsible for the much higher amounts of Na^+ being sent to the shoot (Fig 5.4.a). This scenario is consistent with the observations that root Na^+ content of the KD line was 13% less than that of the WT (Fig 5.4.b). Several studies investigated the physiological role of *AtHKT1* and *SOS3* genes in plant salt tolerance via combinations of knockouts of *Athkt1* and *sos3* in Arabidopsis plants. *AtHKT1* mutations suppressed the Na^+ hypersensitivity of *sos3-1* mutant seedlings (Rus et al., 2001). Since, the *Athkt1sos3-1* double mutant plants were resistant to salt stress regardless to the growth media (Rus et al., 2004; Rus et al., 2001), the s*os3* and *Athkt1* single mutant alleles showed Na^+ hypersensitivity as compared to their wild type (Liu and Zhu, 1998; Mäser et al., 2002a). In contrast, another study showed that *Athkt1* mutations in *sos3* mutant plants did not suppress the Na^+ hypersensitivity of *sos3* mutant plants, but the external Ca^{2+} concentration significantly influenced the salt stress responses of the *Athkt1sos3* double mutant plants (Horie et al., 2006). According to Sunarpi et al. (2005), since the *AtHKT1;1* mutation causes an increase in the Na^+ content of xylem sap, and a corresponding decrease for phloem sap, under salt conditions, it plays an important role in preventing the accumulation of high amounts of Na^+ in leaves. Another study,

by Byrt et al. (2014) found that decreased expression of *TaHKT1;5-D* under varying salt conditions up to 150 mM NaCl, led to an increase of Na^+ in the leaves of wheat, which is considered more salt tolerant than rice. This suggests that TaHKT1;5-D retrieves Na^+ from the xylem vessels of roots, thereby restricting the transportation of Na^+ to shoots, while maintaining a high K^+: Na^+ ratio in the leaves. However, It is important to mention that the studies of Byrt et al. (2014) and Sunarpi et al. (2005) were looking at different alleles of HKTs in plants where the gene and protein are being expressed in the cells the plants would normally express them (stelar tissue). The Sunarpi et al. (2005) worked with knockout plants which had been re-transformed with an *AtHKT1;1* gene under the control of its native promoter, while the Byrt et al. (2014) worked with different alleles of the *TaHKT1;5* gene. Both studies had different levels of HKT activities in the cells they were usually found in.

5.4.3 H^+-ATPase activation regulates the activity of the high-affinity HAKs transporters

The magnitude of salt-induced K^+ leakage has been used as an indicator of salt tolerance in various plant species, such as barley (Shabala et al., 2010), Arabidopsis (Rodrigo-Moreno et al., 2013), pea (Bose et al., 2014), and quinoa (Hariadi et al., 2011). While, salinity induced K^+ efflux in the root zones of both genotypes (Fig 5.8), the highest K^+ efflux was measured in the elongation zone (Fig 5.8.a) and the lowest one in the mature zone (Fig 5.8.b). The rational interpretation of this observation is that the K^+ efflux is the result of dual effects of (1) membrane depolarisation due to Na^+ entry into the cell, with a consequent activation of the depolarisation-activated KOR channel and (2) oxidative stress which causes ROS-activated non-selective cation channels (NSCCs) that, in turn, results in K^+ deficiency in the cytosol and, eventually, cell death. Under salt stress, the KD line attempted to maintain more negative membrane potential across the plasma membrane of epidermal cells via H^+-ATPase activation. Amongst other things, this would provide the driving force for the uptake of potassium. Under conditions of low potassium, the potential candidates for potassium uptake are the HAKs transporters that co-transport K^+ and H^+

(Rodriguez-Navarro, 2000). Thus, higher activity of H^+-ATPase in the KD line resulted in increased potassium uptake, modulated by OsHAKs transporters. Overall, these electrophysiological data support the phenotyping observation that the knocked-down *OsHKT1;5* line showed a higher root K^+ content, thus maintaining a higher root K^+/ Na^+ ratio than that of the WT.

5.4.4 The role of *OsHKT1;5* in the long-distance transport of Na^+ and K^+ from the root to shoot of rice plants

It has been suggested that OsHKT1;5 mediates the unloading of Na^+ from the xylem vessels in the plasma membrane of the xylem parenchyma cells (Ren et al., 2005). Surprisingly, the data showed that when the stellar cells were exposed to salt stress for 30 min, there was no significant difference between the genotypes, with respect to Na^+ influx.

The possible explanation is based on the work of Zamani Babgohari et al. (2013) who reported that an expression of *HKT1;5* was detected earlier in the leaves of wheat than in its roots in response to salt stress. Given the fact that Na^+ accumulation in the shoot builds up over time, in response to salt stress (Munns, 2002), it becomes obvious that the measurements of Na^+ influx into the stellar cells of the roots when taken for only 30 min were not done over a sufficiently long period of time to allow the precise function of OsHKT1;5 in the transport of Na^+, to be identified. This is particularly relevant in the case of stele tissues.

Shabala (2013) have suggested that the beneficial scenario for plants to achieve rapid osmotic adjustment would be to transport Na^+ into the shoot. After this, it would be advantageous to the plants to reduce the rate of xylem Na^+ loading to the absolute minimum to prevent excessive Na^+ concentrating in the photosynthetic tissue. This minimal loading could be achieved by a channel-mediated xylem Na^+ loading, within minutes to hours of salt exposure, and by a thermodynamically active xylem Na^+ loading over a longer period of salt exposure. Two potential

candidates are suggested, namely a SOS1 Na^+/H^+ exchanger (Shi et al., 2002) and a cation-Cl (CCC) co-transporter (Colmenero-Flores et al., 1999). Accordingly, in the case of the present study, because the Na^+ influx was measured in the early stage, immediately after the stellar cells were exposed to salt stress, the result suggests that the effective channel-mediated xylem Na^+ loading leads to increased stele Na^+ loading. This latter is necessary for osmotic adjustment, but at the same time, it also conceals the function of Na^+ uploading via OsHKT1;5, which could occur at slower rate during the first stage of salt stress. Furthermore, Zamani Babgohari et al. (2013) reported that the strongest up-regulation of *TaHKT1;5-D* transcript was detected in wheat plants after 3 h of a 200 mM salt treatment compared to other concentrations tested. On the other hand, since the *OsSOS1* transcript level transiently had increased by 6-fold after 15 h of the salt treatment, measuring Na^+ flux in the stellar cells, immediately after salt treatment was not the ideal way to detect the function of OsHKT1;5 in Na^+ retrieval from the xylem.

The data on K^+ kinetic flux in isolated root stellar tissue showed immediate K^+ efflux upon salt exposure followed by its gradual restoration, although it remained negative, with a non-significant difference between genotypes (Fig 5.8.c). Previous studies have reported that adding 20 mM NaCl to the isolated stellar tissue of barley root caused a rapid K^+ efflux, accompanied by a substantial increase in net H^+ efflux (Shabala et al., 2010). Overall, data concerning ion flux in the stellar tissue did not reveal the physiological role of OsHKT1;5 in the long-distance transport of K^+ and Na^+ immediately after salt treatment.

5.4.5 Higher Ca^{2+} efflux in the KD line might result from the higher NaCl-induced activity of Ca^{2+}-ATPase

Salt stress caused a significant Ca^{2+} efflux in both genotypes (Fig 5.10); however, the KD line revealed a significantly higher Ca^{2+} efflux from the elongation and mature zones compared to the WT (Fig 5.10). Maintaining a low cytosolic Ca^{2+} concentration in plant cells, after the signaling event, requires active efflux pumping of Ca^{2+} from the cytosol. The restoration of basal Ca^{2+} levels

by Ca^{2+}-ATPase and H^+/Ca^{2+}, following a perturbation of cytosolic Ca^{2+} has a beneficial effect on the cytoplasmic metabolism, and can also influence the kinetic and subcellular location of cytosolic Ca^{2+} signals (Tuteja and Mahajan, 2007). Therefore, the speculation is that knock-down of *OsHKT1;5* expression also enhanced the expression level of Ca^{2+}-ATPase, by increasing the efflux of the excess Ca^{2+} from the cytoplasm. This fluctuation in the cytosolic Ca^{2+} could generate Ca^{2+} signature, which can activate the SOS1 pathway, where a myristoylated calcium-binding protein, encoded by SOS3, presumably senses the salt-induced calcium signal (Ishitani et al., 2000). SOS3 interacts with, and activates SOS2, a serine/threonine protein kinase (Liu et al., 2000). SOS2 and SOS3 regulate the expression level of *SOS1,* resulting in increased Na^+ efflux in the elongation zone cells of know-down OsHKT1;5 rather than WT, as shown in the data. A previous study has shown that the transcript level of OsACA6, a Ca^{2+}-ATPase gene in *Oryza sativa,* was enhanced in response to salt and drought stresses, and that the overexpression of the *OsACA6* gene changed several physiological indices, resulting in better plant growth and development , and a higher salt stress tolerance (Huda et al., 2013).

5.4.6 The knock-down of *OsHKT1;5* did not affect the plant growth In rice under drought stress

Osmotic stress is one of the main components of salt stress. Data from the present study showed that both KD and WT genotypes lost more than 35% of their dry weight under water stress (Fig 5.5.a). Plants evolved a number of mechanisms to reduce the effect of such water stress, one of which is osmotic adjustment. This can be achieved through accumulation of compatible solutes, either organic, such as proline, sugar or amino acids, or inorganic, such as K^+ (Shabala and Shabala, 2011). In the study, shoot osmolality of both genotypes increased by 45% in each (Fig 5.6.a), suggesting that the rice plants accumulated either organic and /or inorganic solutes to lower the osmotic potential of their tissues. To investigate this hypothesis, shoot ion content analysis was conducted. The results showed that in drought conditions, shoot Na^+ content did not change under

water stress (Fig 5.6.b), while shoot K^+ content increased significantly by 15%, in each of the genotypes (Fig 5.6.c) This suggests that K^+ may contribute to osmotic adjustment by helping rice plants to maintain plant cell turgor under osmotic stress (Wang et al., 2013). K^+ uptake may be partially mediated by voltage-gated K^+ transporters in the plasma membrane (Shabala and Lew, 2002). The role of K^+ in osmotic adjustment has been studied in rice, where the result showed that adding potassium fertiliser to water-stressed rice plants improve their morphological and physiological parameters (Mohd Zain et al., 2014). However, the contribution of K^+ may not be enough to maintain osmotic adjustment; therefore, glycophytic plants, such as rice, tend to accumulate organic osmolytes, such as proline, sugar and amino acids, as their primary strategy for maintaining osmotic adjustment (Shabala and Shabala, 2011). Studies have suggested that the synthesis of organic osmolytes could come with an energy cost, resulting in dry weight reduction under water stress; this was confirmed by data from the present study. The *OsHKT1;5* gene belongs to the subfamily1, which because it possesses the amino acid, serine (S) in the first pore domain (Mäser et al., 2002b) is permeable to Na^+ only. Thus, this study speculates that OsHKT1;5 might play no role under drought stress. When the two genotypes were compared the data revealed that the KD had a lower dry weight than the wild type both under control and drought conditions. These results suggested that knock-down of *OsHKT1;5* expression had no effect on plant growth in response to drought stress.

5.4.7 ROS-activated K^+ efflux channels could cause cytosolic K^+ pool depletion and increased Ca^{2+} influx that contributes to membrane potential maintenance

Oxidative stress, induced by the hydrogen radical ($OH^\bullet$) and/or hydrogen peroxide (H_2O_2), can activate K^+ efflux through GORK and NSCC (Demidchik et al., 2010). Furthermore, in the present study, it was noted that K^+ efflux developed gradually, and to a lesser degree, under oxidative stress generated by H_2O_2 compared to that generated by ($OH^\bullet$) (Fig 5.11, 12.a). In the present study, both genotypes showed an $OH^\bullet$-induced K^+ efflux from the elongation zone, which

was 8-fold greater than that induced by H_2O_2, with no significant difference between them (Fig 5.11.a, 5.12.a). The ROS-induced K^+ efflux was accompanied by a significant, NSCC-mediated Ca^{2+} influx (Demidchik et al., 2003) from the elongation zone, which, in turn, increased the cytosolic Ca^{2+} which then acts as a second messenger in downstream signaling (Ordoñez et al., 2014). The fact that the KD line showed evidence of a more significant Ca^{2+} influx compared to the WT (Fig 5.12.b), suggests that Ca^{2+} played a signaling role in the KD line. Additionally, it has been reported that the depolarisation effect of Ca^{2+} influx on membrane potential can be reduced by increasing the K^+ efflux (Miedema et al., 2001). Consequently, under oxidative stress, the associated change both in ROS-induced K^+ efflux and Ca^{2+} influx may regulate membrane potential in the elongation zone (Demidchik et al., 2003).

In conclusion, a comparison between a long term Na^+ exposure (greenhouse experiment) and a short term Na^+ exposure (MIFe experiment) firstly revealed that the growth of the KD line when under salt stress was impaired due to extremely high levels of shoot Na^+ and w low shoot K^+ content. These were the result of a high Na^+ loading to the xylem and down regulation of the *OsHKT1;5* activity in the xylem parenchyma in the root of the mutant plants. The result of this was a substantial reduction in the shoot K^+:Na^+ ratio. Secondly, MIFE data show that the KD line performed better than the WT, in terms of its epidermal root cells' ability to exclude more Na^+ from the elongation zone and to reduce K^+ efflux from both zones, as a result of higher activation of the H^+-ATPase. Such activation could possibly be instrumental in enhancing the activity both of SOS1 (the Na^+/H^+ antiporter), and the HAKs K^+/H^+ symporter genes. However, the MIFE experiment was unable to reveal the function of HKT1;5 in Na^+ excluding in rice plants, and it is not representative of what happened during long- term exposure of rice plants to Na^+. As for the drought stress experiment, the phenotyping data revealed no relationship between drought stress and the physiological function of OsHKT1;5.

Chapter 6

General discussion

Both the growth and development of plants are negatively affected by abiotic stress, such as salinity and drought. The severity of these effects is such that, they can cause agricultural production to be greatly reduced. Numerous studies have reported that a plant's salt tolerance is associated with its ability to reduce both Na^+ uptake, and transport, while it is maintaining adequate intracellular K^+ concentration. However, plants evolved a complex network of potassium, sodium transporters, and their regulatory mechanisms to survive, under a biotic stress. For example, Arabidopsis genome contains 75 transport proteins capable of moving K^+ across cellular membranes, of which 35 are highly potassium (Shabala, 2003; Véry et al., 2014; Very and Sentenac, 2002). What is the reason for such diversity? Are some of them redundant? Which one play the major role in maintenance optimal cytosolic K^+/Na^+ ratio under stress conditions? This study have contributed to answering these questions by investigating the physiological role of salt-responsive Na^+ and K^+ transport genes in rice mutants.

6.1 The role of ion channels and transporters in Na^+ uptake under various environmental conditions

Different ion channels have been reported to be involved in Na^+ uptake from the growth medium under deferent environmental condition. One of these, the K^+ inward rectifying channel OsAKT1, could mediate Na^+ uptake under saline condition (Golldack et al., 2003).

Since Na^+ and K^+ have similar ionic radius and hydration energies (Amtmann and Sanders, 1998) Na^+ can potentially fulfil many of the roles that K^+ plays in plant cells, including some of the metabolic one (Maathuis, 2014). So, Na^+ does have a beneficial role in plants, under certain conditions, such as K^+-deficiency, assuming its concentrations do not exceed toxicity freehold. Under this particular condition, Na^+ uptake is important for osmotic adjustment. The present study

identified the positive role of AKT1 in Na^+ uptake, under K^+-deficient conditions. The findings revealed that the addition of 1 and 40 mM NaCl to the bathing medium resulted in a higher Na^+ uptake in *OsAKT1* overexpressors than in the wild type, under conditions of K^+-deficiency and low NH_4^+ concentration (Fig. 4.4a,b). This suggests that, under both conditions, OsAKT1 mediated Na^+ influx. The reasons for this can be found in several studies. For example, Subbarao et al. (2000); Subbarao et al. (1999); Krishnasamy et al. (2014) have reported that elements, like Na^+ and K^+ can replace each other fully in certain non-specific metabolic functions of which osmotic adjustment is one, particularly under K^+-deficent condition. However, the OsAKT1 permeability to uptake Na^+ was inhibited by presence of NH_4^+ in the growth medium under K^+-deficient condition. The data of the present study showed that the presence of 2 mM NH_4^+ in the bathing medium resulted in no significant difference in Na^+ uptake, between the overexpressors and the wild type, when the plants were exposed to 40 mM NaCl (Fig. 4.4 c, d). This finding suggests that NH_4^+ has an inhibitory effect not only on the non-AKT1 component, but also on OsAKT1 under low K^+ conditions.

Despite the fact that Na^+ can have a beneficial role in plants under certain conditions (such as K^+-deficiency), Na^+ has a negative impact on plant growth and development when excessive Na^+ levels in the soil generate osmotic stress and plant also experience Na^+ build-up in their tissues to toxic levels. Demidchik and Maathuis (2007) reported the major portion of Na^+ influx is mediated by NSCCs, which catalyse a passive flux of ions through the plant plasma membrane. On the other hand, high level of Na^+ in growth medium will generate a large electrochemical potential gradient that will favour the passive transport of Na^+ from the growth medium to the cytosol (Blumwald, 2000). Thus, a large Na^+ influx through the NSCC causes a significant membrane depolarisation. On the basis of this view, this study showed that adding 40 mM NaCl to the bath medium resulted in a substantial membrane depolarisation peaking at 3 mins after stress

onset. The MP decreased by 25-30% in the wild type, and *Oshak1*, and *Oshak5* mutants (Fig 3.12), and between 29 and 35 % in the wild type and the *Oshak5* mutant (Fig 3.13).

To detoxify the excess l amount of intracellular Na^+, plants can exclude the Na^+ from the cytosol into the apoplast or store it in the vacuole. Na^+ exclusion is mediated by Na^+: H^+ anti-porters. One of these is SOS1, a plasma membrane Na^+/H^+ exchanger that regulates the Na^+ and K^+ homeostasis in plant cells. *SOS1* expression was reported both for the epidermal cells in the root apex and for the parenchyma cells in the xylem/symplast boundary of roots, stems, and leaves (Shi et al., 2002). In wheat, *Nax* loci affect both the expression and activity of the SOS1-like Na^+/H^+ exchanger (Zhu et al., 2016). Hence, any alteration in the expression level of *OsHKT1;5* would also alter the expression level of *OsSOS1*, which, in turn, would confer Na^+ excluding from the cytosol to the growth medium. The findings of the present study suggest that, upon exposure of rice plants to 80 mM NaCl, knock-down of *OsHKT1;5* expression may have up-regulated the activity PM H^+-ATPase both in the elongation (Fig 5.9a) and mature zones (Fig 5.9b) of the mutant plants, leading to a greater pH gradient across the plasma membrane in the epidermal cells of the roots. This gradient provided the driving force for a plasma membrane SOS1 Na^+/H^+ exchanger to move Na^+ from the cytoplasm to the apoplast. This may be an essential tolerance mechanism, since root tip cells, being predominantly evacuolate, are incapable of vacuolar Na^+ sequestration. Therefore, the cells of elongation zone must rely entirely on extrusion of cytoplasmic Na^+ into the apoplast, via SOS1; this may also explain the insignificant difference in Na^+ flux in the mature zone of the roots. Such a result is consistent with the finding that root Na^+ content of the KD *OsHKT1;5* line was 13 % less than that of the wild type (Fig 5.4.b).

6.1.1 Long distance Na^+ transport

Na^+ unloading from the xylem is one of essential strategies in salt stress physiology (Byrt et al., 2007; Horie et al., 2005; Munns et al., 2012). Glycophytes, such as rice, are mostly

considered to be salt excluders, in order to prevent a significant accumulation of salt in their photosynthetic tissues. OsHKT1;5 is located in the plasma membrane of the parenchyma cells, and it plays an important role in xylem Na^+ unloading, which is considered to be one of a main salt tolerance mechanisms in plants (Platten et al., 2013). Furthermore, Cotsaftis et al. (2012) reported that root-to-shoot Na^+ transfer is controlled both by *OsHKT1;5* transcript levels and protein structure, while sheath-to-blade Na^+ transfer is controlled by *OsHKT1;4* transcript levels and alternative splicing. Consistently, the finding of this study was that the KD line accumulated a higher concentration of Na^+, accompanied by a lower shoot K^+: Na^+ ratio compared to the WT (Fig. 5.4). Because of this, growth reduction in the KD line was greater than in the WT, under 80 mM NaCl (Fig. 5.2). This reduction was due to not only to higher shoot Na^+ concentration in the KD line, but also to the fact that this line also had a lower shoot K^+ concentration. Taken together, the K^+:Na^+ ratio was lower in the KD line than in the WT. Therefore, the result revealed the critical role of OsHKT1;5 in maintaining optimal shoot K^+:Na^+ ratio in the rice plants under salinity stress (Fig. 5.2).

The *OsHKT1;5* gene because it possesses the amino acid serine (S) in the first pore domain (Mäser et al., 2002b), it is permeable to Na^+ only. Thus, the present study speculates that OsHKT1;5 possibly plays a role in plant growth, under drought stress. The finding of this study showed that the KD significantly accumulated less dry weight as compared to the wild type both, under control and drought conditions (Fig. 5.5). These results suggested that knock-down of *OsHKT1;5* expression had no effect on plant growth in response to drought stress. However, the root Na^+ content of the KD line was lower as compared to the wild type (Fig. 5.6d). Thus it can be assumed from this observation that knock-down of *OsHKT1;5* expression increased the expression of *OsSOS1*, resulting in more Na^+ efflux.

6.2 The role of K^+ transporters and channels in the maintenance of intracellular K^+ homeostasis under various abiotic stresses

Potassium is an essential macronutrient required by plants for growth and development. It is, widely, used as a fertiliser to increase plant production, since the K^+ concentration in agricultural soils is often low falling within the micro-molar range (Clarkson, 1985). Therefore, a study that examines the uptake of K^+ within the high-affinity range of concentrations is essential for three main reasons: firstly, because of the need to increase K^+ use efficiency particularly, under a K^+-deficient condition so as to improve yields; secondly, because improving plant K^+ nutrition would reduce the cost of using K^+ fertilizer, thereby making it a cost-effective option for the agricultural industry; and lastly, because improving plant K^+ nutrition will improve plant salt tolerance, and so permit more land to be used for agriculture. This present study demonstrated that both *OsHAK1* and *OsHAK5* genes play a crucial role in K^+ uptake, under a high-affinity K^+ system in rice plants. The results of the present study showed that salinity caused membrane depolarisation (Fig. 3.12). Several prior studies have reported that such membrane depolarisation induces K^+ efflux, via outward-rectifying depolarisation-activated K^+ channels GORK and ROS-activated channels NSCC (Demidchik et al., 2010; Shabala, 2009; Shabala and Cuin, 2008). The present study demonstrated that this K^+ efflux was induced from the elongation and the mature zone of the mutant plants, as well as, in the WT by the addition of 40 mM NaCl to the measuring bath. In addition to this, the loss of function of *Oshak1* and *Oshak5*, under the K^+-deficient condition, resulted in an immediate K^+ efflux that was 3-4 times higher in the mutant plants than in the WT (Fig. 3.1). Chen et al. (2015) reported that *OsHAK1* was expressed particularly in the epidermal and vascular cells of rice roots, where its expression was much stronger in both the tip of the preliminary root and in the lateral roots and that it contributed to about 50-55% of the high-affinity K^+ uptake, under a K^+-deficient condition during the short-term growth of rice plants. Under the same conditions, Yang et al. (2014) has also found that loss of function of *Oshak5* reduced the net

K^+ uptake up by 20%. It is concluded, therefore, that under the K^+-deficiency conditions, the loss of function of *Oshak1* and *Oshak5* caused further K^+ efflux from the elongation zone (Fig. 3.1a) but not from the mature zone (Fig. 3.2a), thereby shedding light on the critical role played by OsHAK1 and OsHAK5 in K^+ homeostasis and, thus in plant nutrition both, under the K^+-deficient and salt condition. Under low-affinity K^+ uptake, there was no difference in K^+ flux between the mutant plants and the WT (Fig. 3.1b, 3.2b). This suggested that K^+ influx, under high-affinity K^+ uptake, is consistently mediated by transporters, such as HAK, while under low-affinity K^+ uptake, the K^+ influx is passively mediated by channels (Alemán et al., 2011; Maathuis and Sanders, 1996). These channels can thermodynamically catalyse downhill fluxes, that are three orders higher than those catalysed by pumps and transporters (Tester, 1990).

AKT1 has been shown to mediate K^+ uptake from both low and high-affinity potassium uptake systems (Gierth et al., 2005; Hirsch et al., 1998; Spalding et al., 1999). The growth of *akt1-1*mutants was inhibited under K^+-deficient condition with regard to the presence of NH_4^+ (Spalding et al., 1999). The present study showed that the overexpressoion of *AKT1* resulted in a higher K^+ influx than in the wild type when just 1 mM NaCl was added, and under a low NH_4^+ concentration condition (Fig. 4.5a). This result confirms that OsAKT1 is a K^+ potassium channel and that it uptakes Na^+ in the presence of a low K^+ and NH_4^+ concentration. This function of OsAKT1 was diminished when the root was exposed to 40 mM NaCl, and surprisingly there was no significant difference between the genotypes. The result of this study, in agreement with a previous study was conducted by Spalding et al. (1999) who reported that the K^+ permeability of AKT1 was between 55-63% in the absence of NH_4^+, and with an external K^+ concentration of 10-1000 µM.

6.3 The signalling role of the calcium sensor CBL-CIPK9 in intracellular K^+ homeostasis in response to hostile environmental conditions

Studies by Pandey et al. (2007) and Liu et al. (2013) showed that CIPK9 plays an important role in K^+ homeostasis in Arabidopsis, particularly, under a K^+-deficient condition. Additionally, the expression of *CIPK9* was induced by several abiotic stresses including osmotic, high salinity, cold and wounding. However, the findings of two studies for the exact functional role of CIPK9 in Arabidopsis, under the K^+-deficient condition were diametrically opposed. Pandey et al. (2007) found that, although the loss of function of *cipk9* did not have any effect on the K^+ uptake and content of the *cipk* mutant compared to the wild type, the hypersensitivity of the mutant increased, under the K^+-deficient condition. In contrast, (Liu et al., 2013) showed that in response to K^+-deficient condition, the loss of function of *cipk9* resulted in a tolerant phenotype compared to the wild type in Arabidopsis. The results of the present study showed that the loss of function of *Oscipk9* did increase plant sensitivity, under the K^+-deficient condition, while the only phenotyping difference occurred when the plants were grown, under the K^+-deficient condition. Thus, this finding suggests that OsCIPK9 does play an important role in K^+ homeostasis, under the K^+-deficient conditions. A further study was conducted, under a saline condition, to investigate the role of OsCIPK9 in Na^+ homeostasis. The results of this experiment showed that *Oscipk9* mutants maintained a lower shoot K^+: Na^+ ratio compared to the WT (data are calculated based on the data presented in Fig. 2.3). This lower ratio was due to low shoot K^+ content (Fig 2.3c). However there was no significant difference in the shoot Na^+ content of the mutant compared to that of the wild type (Fig. 2.3a). This implies that OsCIPK9 plays a critical role in K^+ nutrition signaling but not in Na^+ signaling, particularly under the K^+-deficient condition, thereby opening a new horizon in the understanding of salt stress and K^+ nutrient deficiency signaling in rice plants.

6.4 The role of Ca^{2+} signature in regulating intracellular K^+ homeostasis in response to oxidative stress

Ca^{2+} plays an important role in plant stress responses, as it is known to be a ubiquitous intracellular second messenger molecule that is involved in many signal transduction pathways in plants (Tuteja, 2009). The intracellular Ca^{2+} can be changed by many environmental stimuli, acting via Ca^{2+} channels, pumps, and transporters located in the plasma membrane of plant cells to generate what is called Ca^{2+} signature (Tuteja and Mahajan, 2007). This signature can be detected by a number of calcium sensors. Among of these, the calcineurin-B-like protein (CBL)-CBL-interacting protein kinase (CIPK) complex plays a critical role in modulating the expression of the targeted genes, as a response to environmental stimuli (Manik et al., 2015). The results of the present study showed that there were three factors that played an important role in K^+ homeostasis, as a response to oxidative stress. These factors are the extracellular concentration of K^+ and Ca^{2+}, and the expression level of *OsCIPK9* in the mutant plants. By taking into account these factors, the study made three observations. The first of these was that when the rice plants were cultivated in a medium in which the concentration of Ca^{2+} was high and that of K^+ was low, the *Oscipk9* mutant plants showed both a higher K^+ efflux (Fig. 2.8a) and Ca^{2+} influx (Fig. 2.9a) than the WT, when H_2O_2 was added. These results suggested that the application of H_2O_2 mediated K^+ efflux through GORK (Demidchik et al., 2010) and Non-Selective Cation Channels (NSCCs) (Pottosin et al., 2012), and Ca^{2+} influx, through NSCCs (Demidchik and Maathuis, 2007). The increase in the concentration of intracellular Ca^{2+} (Ca^{2+} signature) can be used by plants to produce a signal to modulate the expression of stress-responsive genes. However, in the mutant plants, the loss of function of *Oscipk9* the Ca^{2+}-sensor required to intercept the Ca^{2+} signal and to modulate the expression of K^+ transporters caused them to experience K^+ efflux that was higher as compared to the WT where the expression level of *OsCIPK9* is normal. Under K^+-deficiency conditions, the only potential target for OsCIPK9 was the high-affinity potassium transporters. The second

observation was that the low extracellular Ca^{2+} concentration caused a further K^+ efflux in the *Oscipk9* mutant plants, but triggered no change in K^+ efflux in the WT (Fig. 2.8a) compared to the plants grown, under both a high Ca^{2+} and low K^+ conditions This finding suggests that the extracellular Ca^{2+} concentration may have mitigated the negative effect of the oxidative stress that was generated by H_2O_2. The third observation is that in the presence of a high external K^+, the addition of H_2O_2 resulted in no significant difference in K^+ flux response between the lines (Fig. 2.8c, d). As it is under this same condition that low-affinity K^+ transporters also function in the root, this result suggests that these are not potential targets for OsCIPK9. Furthermore, the function of the low-affinity K^+ transporters may have concealed not only the K^+ efflux that was induced by H_2O_2, but also the difference in K^+ flux between the mutant plants and the WT, which observed as a result of the loss of function of *Oscipk9* in the mutant plants.

Another role of Ca^{2+} as a second messenger was found in response of *OsHAK1* and *OsHAK5* genes to oxidative stress (Chapter 3). The result of this study showed that adding 10 mM H_2O_2 into the bathing medium significantly increased Ca^{2+} (Fig 3.9a, c) and K^+ (Fig 3.5a, c) influx in the elongation zone of the wild type as compared to the *Oshak1* and *Oshak5* mutant plants. This result was against our expectation, because the loss of function of *Oshak1* and *Oshak5* would enable these mutants to compensate for the K^+ efflux that is caused by oxidative stress. Further investigation was done on OsRbohs proteins encoding NADPH oxidases that play an important role in the signaling network of ROS production in plants (Baxter et al., 2014). the expression levels of *OsRbohA, B,* and *C* were 7-14 times higher in the wild type than in either the *Oshak1 or the Oshak5* mutants, while the expression level of *OsRbohG* was lower in the *Oshak5* (DJ) and higher in the *Oshak5* (HY) mutant as compared to the expression level of *RbohG* in the wild type, suggesting that loss of function of *Oshak1* and *Oshak5* down-regulated the expression level of *OsRboh*s, under the low K^+ condition. Consequently, an increase in the intracellular Ca^{2+} concentration may directly activated or indirectly (via Ca^{2+} sensors) phosphorylated the OsRboh

proteins, which in turn caused higher production of H_2O_2 in the wild type as compared to the *Oshak1* and *Oshak5* mutants. Thus, excessive level of H_2O_2 triggered more K^+ efflux via NSCCs and GORK in the wild type as compared to the *Oshak1* and *Oshak5* (Shabala and Pottosin, 2014).

6.5 The OsAKT1 channel is involved in NH_4^+ uptake

Various studies have consistently reported that the presence high concentration of NH_4^+ in the growth medium reduces K^+ uptake and accumulation in rice plants (Szczerba et al., 2008a; Wang et al., 1996). The present study has identified that increasing the external K^+ concentration could potentially have effect on NH_4^+ uptake in view of the fact that K^+-based fertilisers are being used extensively and in ever increasing amounts in a bid to make agricultural land more productive. The finding of this study displayed that stronger NH_4^+ influx was stimulated in the elongation zone of *OsAKT1* overexpressor as compared to the wild type (Fig. 4.2a), when 1mM K^+ was added to the bathing medium. On the other hand, the loss of function of the *akt1* gene in the *akt1* KO mutant plants resulted in no response to the K^+ treatment (Fig. 4.2b). These results confirm that NH_4^+ influx is mediated by the Shaker-type potassium channel, OsAKT1 in rice plants, when the external K^+ concentration changes from high affinity range to low affinity range, and are in agreement with the finding of ten Hoopen et al. (2010) who reported that the net NH_4^+ influx doubled when both K^+ and NH_4^+ were present compared to in the presence of NH_4^+ alone. However, Szczerba et al. (2008a) reported that NH_4^+ influx reduced by 60%, and cytosolic NH_4^+ by 3-4 times, when the external K^+ concentration changed from the high affinity range to low affinity range. Overall, the present study confirms that OsAKT1 is involved in short-term NH_4^+ uptake, particularly when the K^+ concentration is increased to a point where it is within the low-affinity K^+ uptake range. Therefore, excessive use of NH_4^+ fertilisers may cause NH_4^+ toxicity by increasing NH_4^+ uptake via K^+ transporters, under low K^+ conditions. The discovery of this role played by OsAKT1 in NH_4^+ uptake highlights the importance of judiciously managing the use of

fertilisers to increase agricultural yields particularly when the crop plants in question are under low K^+ conditions.

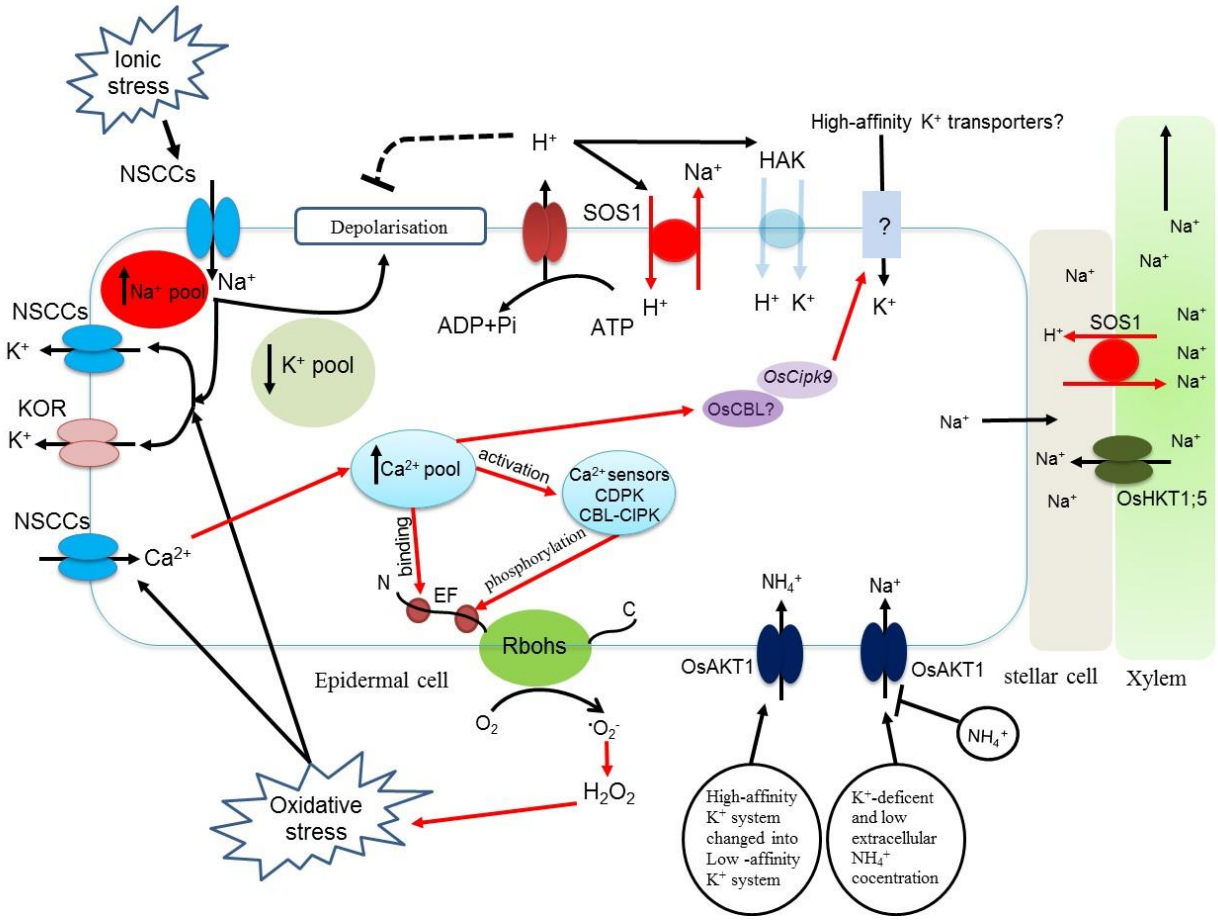

Figure 6. 1 The proposed model explaining the ionic, osmotic and oxidative components of salinity stress in the epidermal and xylem parenchyma cells of rice roots of the mutant and WT lines.

The following model may be suggested explaining the ionic, osmotic and oxidative components of salinity stress in the epidermal and xylem parenchyma cells of rice roots of the mutant and WT lines (Fig 6.1). Exposure to 80 mM NaCl may cause a depolarisation of the plasma membrane, resulting in a massive Na^+ influx from the medium to the epidermal cells via non selective cation channels (NSCCs), accompanied by a marked K^+ efflux via K^+-outward rectifying channels (KOR) and NSCCs. This caused depletion in the cytosolic K^+ pool and an increase in the Na^+ going into xylem parenchyma cells. Since the activation of H^+-ATPase energizes both the SOS1, Na^+/H^+ exchanger and the HAK, a K^+/H^+ symporter. Oxidative stress brought by salinity

induced a massive K^+ efflux accompanied by a huge Ca^{2+} influx via NSCCs. In the xylem parenchyma, knock-down of *OsHKT1;5* expression caused increased accumulation of Na^+ in shoot tissue by altering the function of SOS1.

The uptake of K^+, NH_4^+, and Na^+ by OsAKT1, showing that the occurrence of the Na^+ uptake by OsAKT1 depends on the K^+ electrochemical potential gradient. Extracellular NH_4^+ inhibited Na^+ uptake by OsAKT1. This model also shows that the addition of 1 mM K^+ to the bathing medium stimulated the NH_4^+ uptake by OsAKT1. Under both K^+-deficiency and low extracellular NH_4^+ and Na^+ concentrations conditions, OsAKT1 mediated K^+ uptake in response to Na^+ treatment. Application of H_2O_2 caused K^+ efflux via GORK (Demidchik et al., 2010) and NSCCs (Pottosin et al., 2012), and Ca^{2+} influx via NSCCs (Demidchik and Maathuis, 2007). The increase in intracellular Ca^{2+} generated Ca^{2+} signature, which was detected by the Ca^{2+}-sensor protein, OsCIPK9. This protein activates the downstream target. In this study, the high-affinity K^+ transporters were the potential target of OsCIPK9. The loss of function of *Oshak1* and *Oshak2* decreased the expression of *Rbohs* genes led to reduce the production of H_2O_2, which in turn causes less K^+ efflux in the *Oshak1* and *Oshak5* mutant lines compared to the wild type.

Future work

The final objective of this study was to understand the main mechanisms of salt tolerance in rice in order to improve rice production in salt affected areas. While some significant findings have been achieved in this study, several questions still remain and these need further investigation.

This study identified the possible role played by OsCIPK9 in the calcium signaling network in rice plants, in response to oxidative stress, that it may regulate the activity of other protein components involved in the response to K^+-deficiency. Of a particular note is the fact that K^+ efflux was altered in the *Oscipk9* mutant, as this suggested that the downstream target of *OsCIPK9* may be directly related to K^+ uptake transporters, under the high-affinity K^+ range. Therefore, it is recommended that further study be undertaken to identify the target protein of OsCIPK9 either by generate a library of protein fragments of potential K^+ transporters, to test the interaction between Potential K^+ transporters, with OsCIPK9 in a yeast 2 hybrid system, or an antibody could be developed to OsCIPK9 and used in a protein pull down system to try and captures OsCIPK9, when it is interacting with its target in a column.

Once interacting transporters have been identified the generation of transgenic plants with split YFP constructs (such as the SPYCE SPYNE system) (Walter et al., 2004) would show whether the interaction of the CIPK9 with the potential target occurs *in planta*, *in situ* RT-PCR would show if the genes are expressed in the same cell type. Then, the electrophysiological study could be performed on oocytes, expressing the downstream target with and without OsCIPK9 to determine if the activity of the target protein changed.

This study also identified the transient increase in cytosolic Ca^{2+} as being critical to the induction of a physiological response to oxidative stress. In addition, it also established that exogenous Ca^{2+} lessened the negative effect of H_2O_2 on the K^+ homeostasis. Therefore, it is

important that future studies investigate the electrophysiological role of OsCIPK9 in K^+ homeostasis under a wide range of exogenous Ca^{2+} concentrations and K^+-deficient conditions.

Plant adaptation to different environmental conditions and nutrient availability requires a precise regulation of the transport systems involved in nutrient uptake. Therefore, it is anticipated that the transporters are modulated at the transcriptional and/or posttranscriptional level. The present study established that the high-affinity K^+ transporters, HAK5, and HAK1 are critical for plant growth, as they are involved in K^+ uptake under K^+-deficient condition. In Arabidopsis, Ragel et al. (2015) reported that CBL-CIPK23 complex regulates the HAK5-mediated high-affinity K^+ uptake. Therefore, for better understanding of the regulatory mechanisms of both OsHAK1 and OsHAK5 in K^+ uptake under K^+-deficient condition, to study the regulatory proteins of OsHAK1 and OsHAK5 transporters in rice plants.

This study revealed that OsAKT1 was involved in Na^+ and NH_4^+ uptake at cellular level under a K^+-deficient condition during a short-term experiment. Additionally, it established that the Na^+ uptake by OsAKT1 was inhibited by the presence of a high concentration of NH_4^+ in the growing medium. Therefore, it is recommended that future studies must conduct a growth experiment involving the *Osakt1* mutants and wild types to test the long-term effect of various NH_4^+ concentrations on the ability of OsAKT1 to uptake K^+ and Na^+, under a K^+-deficient condition. The knowledge thus gained may serve to make the use NH_4^+ fertilizer, within the agricultural industry, in a more cost-effective manner.

Finally, the present study showed that knock-down of *OsHKT1;5* expression affected both K^+ and Na^+ flux, as well as tissue ion content. Therefore, it is feasible to suspect that it may also affect the expression of other transporters, such as OsSOS1, Ca^{2+}-ATPase and H^+-ATPase, as has been speculated in the present study. To test the hypothesis that *HKT1;5* could affect *SOS1* expression, a qRT-PCR analysis has to be done to check the SOS1 expression. Since several studies have evaluated the effects of different combinations of *SOS1* and *HKT1* mutants on plant

phenotype in Arabidopsis (Horie et al., 2006; Liu and Zhu, 1998; Mäser et al., 2002a; Rus et al., 2004; Rus et al., 2001), it is worth generating, within the same rice plant, double mutants of these genes, such as *sos1* and *hkt* double knockouts, or *sos1* and over-expression of HKT1;5. This may help identify the link between the processes -taking place in this study. However, further study of the ways in which transporter proteins, such as SOS1, Ca^{2+}-ATPase, HAK, AKT1, GORK and NSCC, altered in this mutant, could only serve to deepen the current understanding of how OsHKT1;5 influences ion uptake and homeostasis in rice plants.

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Supporting information

Statement

For the benefits of readers in order to understand the results of this study, the expression analysis of targeted genes (*HAK1*, *HAK5*, *AKT1*, and *HKT1;5*) is given below. This data was obtained from the published work of our research collaborators from UK and China who have provided seeds for electrophysiological studies. More details are available in the following papers:

- Chen, G., Hu, Q., Luo, L., Yang, T., Zhang, S., Hu, Y., Yu, L., and Xu, G. (2015). Rice potassium transporter OsHAK1 is essential for maintaining potassium-mediated growth and functions in salt tolerance over low and high potassium concentration ranges. *Plant, Cell & Environment* **38**, 2747-2765.
- Yang, T., Zhang, S., Hu, Y., Wu, F., Hu, Q., Chen, G., Cai, J., Wu, T., Moran, N., Yu, L., and Xu, G. (2014). The role of a potassium transporter OsHAK5 in potassium acquisition and transport from roots to shoots in rice at low potassium supply levels. *Plant Physiol* **166**, 945-959.
- Ahmad, I., Mian, A., and Maathuis, F. J. M. (2016). Overexpression of the rice AKT1 potassium channel affects potassium nutrition and rice drought tolerance. *Journal of Experimental Botany* **67**, 2689-2698.
- Kobayashi, N. I., Yamaji, N., Yamamoto, H., Okubo, K., Ueno, H., Costa, A., Tanoi, K., Matsumura, H., Fujii-Kashino, M., Horiuchi, T., Al Nayef, M., Shabala, S., An, G., Ma, J. F., and Horie, T. (2017). OsHKT1;5 mediates Na^+ exclusion in the vasculature to protect leaf blades and reproductive tissues from salt toxicity in rice. *The Plant Journal*.

The data of *Oscipk9* mutant plants were obtained via personal communication with our collaborator in Korea.

DNA and *Ds* insertion for *Oscipk9* mutant plants

Oscipk9 mutants were obtained from *Ds* and T-DNA insertion populations. Trap *Ds* and T-DNA were inserted in the first, and 11^{th} intron, respectively, of *OsCIPK9* (Fig S1.a). Northern hybridisation showed that transcripts were not detected with DNA sequences downstream of the

Ds or T-DNA insertion site in the mutant lines (Fig. S1.b). Six revertant alleles were obtained by remobilizing *Ds* from *OsCIPK9::Ds*. Base changes took place during excision of Ds (Fig. S1.c). The base alterations in the intron did not disrupt the splicing of transcripts in revertant alleles, so they showed the same phenotypes as normal siblings.

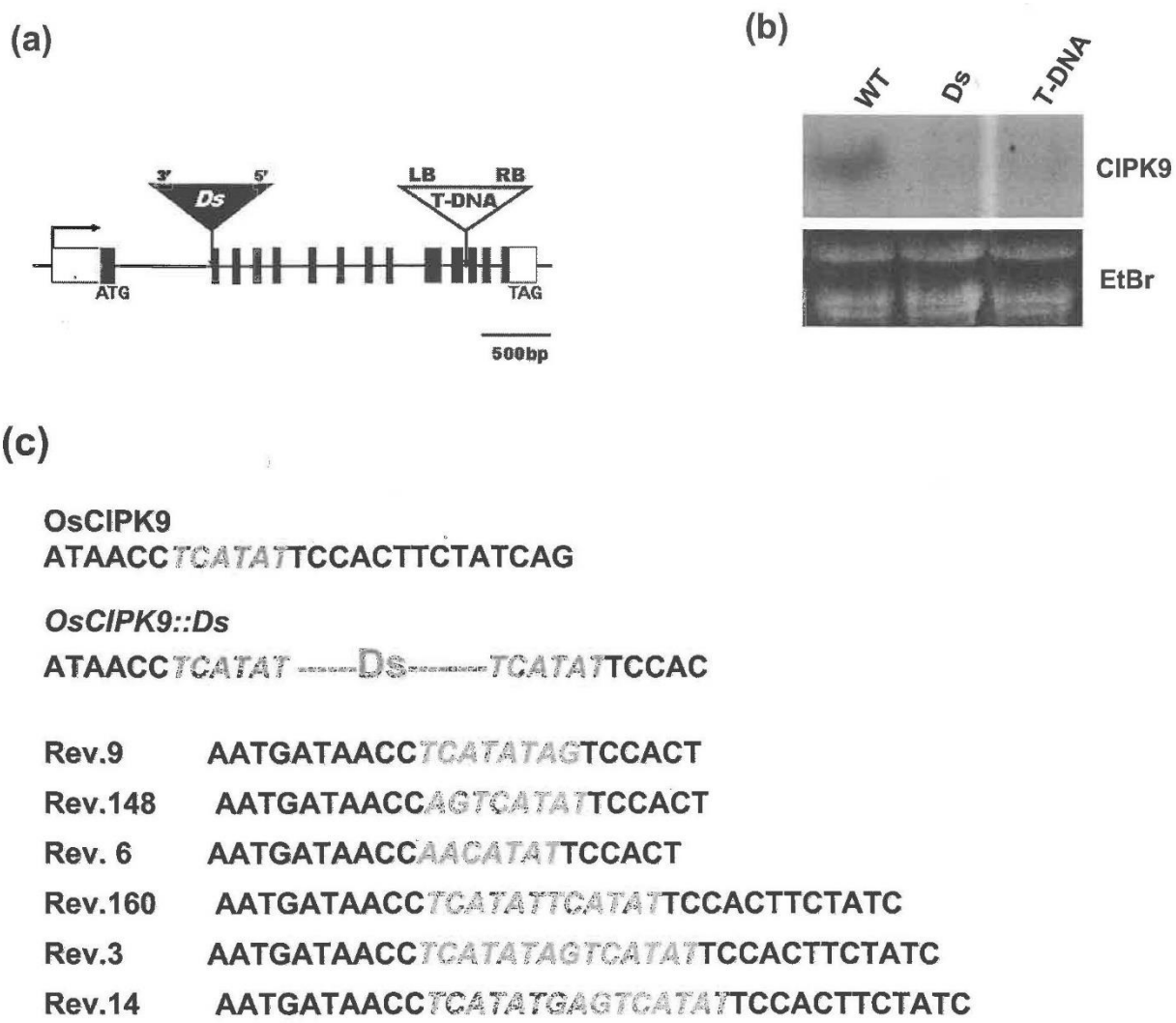

Figure S1 *Oscipk9* mutants and revertants. (a) *Oscipk9* mutants were obtained from *Ds* and T-DNA insertion populations. Trap *Ds* was inserted near the 3' end of the first intron of *OsCIPK9*. T-DNA was inserted into the 13th exon of *OsCIPK9*. (b) Northern hybridisation. The probe was a DNA sequence downstream of the *Ds* or T-DNA insertion site. It was not detected in *Ds* and T-DNA *Oscipk9* mutant lines. (c) revertant alleles. Six revertant lines were derived from *Ds* excisions at the *Oscipk9::Ds* locus. Base changes in intron 1 of these revertant alleles are listed in (Fig S1).

[6]Identification of *oshak5* transfer DNA insertion mutant lines

The identification of two putative transfer DNA (T-DNA) insertion lines of *OsHAK5* knockout mutants were done by searching the SIGNAL database (http://signal. salk.edu/cgi-bin/RiceGE). Their genetic backgrounds were cv HY and cv DJ. The integration positions were verified by sequencing the right border of PCR products, with the primers used to identify the T-DNA mutants (Table S1). One mutant of the cultivar cv HY and another mutant of cv DJ, were identified with only one copy of the insertion, and the insertional positions were consistent with the supplier's report. qRT-PCR analysis of mRNA from K-starved roots and shoots of the T2 generation were used to confirm these two *OsHAK5* KO lines. There was no detectable *OsHAK5* transcript in either knockout mutant (Fig. S2).

Table S1 The primers were used to identify two homozygous mutant lines of *Oshak5*

Table S1 has been removed for copyright reasons, as has page 248 which contains images, and figure S2 on page 249. The removed content can be found in the published article: Yang, T., Zhang, S., Hu, Y., Wu, F., Hu, Q., Chen, G., Cai, J., Wu, T., Moran, N., Yu, L., Xu, G. (2014). The role of a potassium transporter OsHAK5 in potassium acquisition and transport from roots to shoots in rice at low potassium supply levels, Plant Physiol 166, 945-959. DOI: 10.1104/pp.114.246520

[6] This study was carried out by Yang et al. (2014).

Figure S3 and supporting text has been removed for copyright reasons. The removed content can be found in the published article: Chen, G., Hu, Q., Luo, L., Yang, T., Zhang, S., Hu, Y., Yu, L., Xu, G. (2015). Rice potassium transporter OsHAK1 is essential for maintaining potassium-mediated growth and functions in salt tolerance over low and high potassium concentration ranges. Plant, Cell & Environment, 38, 2747-2765. DOI 10.1111/pce.12585

Figure S3 Identification of *Oshak1* mutant line

[7] This study was carried out by Chen et al (2015).

Pages 251-254 contain figures S4 and S5 and supporting documentation which have been removed for copyright reasons. The removed content can be found in the published article: Ahmad, I., Mian, A., Maathuis, F. J. M. (2016). Overexpression of the rice AKT1 potassium channel affects potassium nutrition and rice drought tolerance, Journal of Experimental Botany 67, 2689-2698. DOI 10.1093/jxb/erw103

[8] This study was carried out by Ahmad et al. (2016).

Pages 255-256 contain figure S6 and supporting documentation which have been removed for copyright reasons. The removed content can be found in the published article: Kobayashi, N. I., Yamaji, N., Yamamoto, H., Okubo, K., Ueno, H., Costa, A., Tanoi, K., Matsumura, H., Fujii-Kashino, M., Horiuchi, T., Al Nayef, M., Shabala, S., An, G., Ma, J. F., Horie, T. (2017). OsHKT1;5 mediates Na+ exclusion in the vasculature to protect leaf blades and reproductive tissues from salt toxicity in rice, The plant journal, 91(4), 657-670 DOI: 10.1111/tpj.13595

[9] This study was carried out by (Kobayashi et al.2017).